The

Structure and Function of

MUSCLE

Volume II

Volume I: Structure

Volume II: *Biochemistry and Physiology*

Volume III: Pharmacology and Disease

The Structure and Function of MUSCLE

Edited by

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Volume II

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PREFACE

In this volume we deal mainly with the functional rather than the structural aspects of muscle. However these two aspects are indissoluble. Those who have perused Volume I will have noted a constant referral of structure to function and in this volume a referral of function to structure will be noted. Dr. Andrew Szent-Györgyi's initial chapter in fact deals with the nature of the actual structural units of muscle—the proteins. In the other chapters the detailed biochemical activity of structures particularized in Volume I is given. Physiological aspects of muscle action and functioning of muscle as part of an integrated organism mediated by the central nervous system and the endocrines and affected by temperature, fatigue, and training all find a place within the pages of Volume II.

This particular volume will be of special interest to biochemists and physiologists and also to neurologists, pathologists, cardiologists, and others concerned with neuromuscular disease in humans and animals, since it provides the norms from which muscular action deviates in pathological conditions.

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February, 1960

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CHAPTER I

Proteins of the Myofibril

ANDREW G. SZENT-GYÖRGYI

I. INTRODUCTION

A. PROTEINS OF MUSCLE

The proteins of muscle may be conveniently grouped into four major fractions. These fractions are: the sarcoplasmic proteins, the proteins of the granules, the proteins of the myofibril, and the stroma proteins. Such a grouping is convenient, since the members of the different fractions are localized in different entities of the muscle cell and contribute in a different fashion to its various activities. The differences in the extractability of these major fractions is one of the bases of such a classification.

1. *The Sarcoplasmic Proteins*

The sarcoplasmic proteins are extracted with the greatest of ease and are frequently mentioned as the "soluble proteins" of muscle. These proteins occupy mostly the space between the myofibrils and can be brought into solution readily with water or with neutral salt solutions of low ionic strength ($\Gamma/2 < 0.2$). The solution thus obtained has a low viscosity. The proteins extracted are myoglobin and enzymes, including

all the components of the glycolytic system and the various phosphokinases. The sarcoplasmic proteins do not contribute significantly to the filamentous organization of muscle, and after their removal, the characteristic morphological features of the different types of muscles remain apparently unaltered. In fact, these features can be best resolved in muscle preparations from which the soluble proteins have been previously extracted. The sarcoplasmic proteins do not seem to be directly involved in the structural reorganization which results in contraction. Their function is directed to other, mainly metabolic, activities of the cell. The contribution of sarcoplasmic proteins to the total proteins of muscle varies from species to species and depends on the embryonic development of the cell. The variation in the soluble proteins is considerably greater than the variation in fibrillar proteins. In adult rabbit or chick striated muscles, about 30% of the total protein is of sarcoplasmic origin (Hasselbach and Schneider, 1951; Robinson, 1952a, b). In the early stages of development (14 days), the sarcoplasmic proteins may comprise nearly 70% of the total proteins of embryonic chick muscle.

2. *Granules*

Most of the granules are removed from a well-homogenized muscle together with the sarcoplasmic proteins, by solvents of low ionic strength. Differential centrifugation is a convenient way to separate them from the soluble proteins. The important components of this fraction are the nuclei, the sarcosomes or mitochondria, and the microsomes. Several important activities are associated with these particles. The sarcosomes carry the enzyme systems of the oxidative cycle. The amount present in different muscles varies widely, and there is a good correlation between oxidative activity and sarcosomal content of various muscles (Paul and Sperling, 1952; Chappell and Perry, 1953). The important "relaxing factor" (Marsh, 1951) is closely associated with the microsomal particles (Kumagai *et al.*, 1955; Portzehl, 1957). The magnesium-activated ATPase of muscle which is not myosin appears to originate also from the microsomal fraction (Kielley and Meyerhof, 1950). The granules are localized between the myofibrils and in some instances appear to be localized in register with the Z membranes (Perry, 1956). Their presence is not a necessary requirement of contraction, although some of their components or products may significantly modify the behavior of the proteins participating in con-

traction, and may thus exert a controlling function. Elucidation of their role will certainly help to bridge the gap between the *in vitro* contraction of extracted fibers and that of the intact muscle fiber.

3. *Myofibrillar Proteins*

These are the proteins which are responsible for the filamentous organization of muscle and which directly participate in contraction. Their removal is accompanied by the disorganization and disappearance of the myofilaments (Hasselbach, 1953; Hanson and Huxley, 1953), and by a complete loss of birefringence. The myofibrillar proteins are frequently denoted as the "structure proteins" or "insoluble proteins" of muscle. For their extraction, neutral salt solutions of high ionic strength ($\Gamma/2 > 0.5$) are required, even though, after extraction, some of them are soluble at lower ionic strength. The resistance to extraction is partly a result of the intimate associations and interactions between these proteins within the myofilaments. The high viscosity of the extract indicates the fibrous nature of the proteins brought into solution. From myofibrils of striated muscles and from most smooth muscles, three well identified components can be isolated: myosin, actin, and tropomyosin. In the "catch" muscles of Molluscs and Annelids, there is an additional major component—paramyosin. Actin, myosin, and tropomyosin comprise about 80% of the proteins of the myofibril of rabbit skeletal muscle (Perry, 1956). Since these structural proteins can be extracted under conditions which are sufficiently mild that they retain their biological activities, and since the solubilized proteins do not show obvious signs of denaturation, they are uniquely suited for study of some of the general properties of fibrous proteins, apart from the specific question of their participation in contraction.

4. *Stroma Proteins*

These are the proteins retained in the residue after prolonged extraction of a well-homogenized muscle with strong salt solutions. The residue contains some material of a collagenous nature contributing to the structure of the sarcolemma and possibly to the Z membrane. Because of their poor extractability, our knowledge of the components of stroma proteins is rather limited and their characterization has hardly begun.

B. Terminology and Reviews

This chapter will describe some of the properties of the structure proteins of muscle. Since their role in contraction and their contribution to the structure of the muscle cell is discussed in other chapters, emphasis will be laid upon their isolation, characterization, and properties. Actin, myosin, tropomyosin, and paramyosin will be discussed in some detail. Apart from these, a number of other proteins, presumably of myofibrillar origin, have been reported. These are: delta protein (Amberson *et al.*, 1957), metamyosin (Raeber *et al.*, 1955), contractin or Y protein (Dubuisson, 1948; Schapira *et al.*, 1957), and X protein (A. G. Szent-Györgyi *et al.*, 1955). The identification and characterization of these proteins and their possible relationship to the major group of myofibrillar proteins is still in an initial stage, and they will be dealt with only cursorily.

The following nomenclature will be used:

Myosin denotes preparations free of actin or poor in actin. It includes the "crystalline" myosin of Szent-Györgyi (1943), the L-myosin of Weber (Schramm and Weber, 1942), and myosin A, a relatively actin poor preparation (Banga and Szent-Györgyi, 1941). The myosin β electrophoretic component of Dubuisson (1946a, b) corresponds to myosin.

Actomyosin denotes the myosin B of Szent-Györgyi (Banga and Szent-Györgyi, 1941) obtained by direct extraction from muscle, and the "artificial" or "synthetic" actomyosin prepared by mixing actin and myosin solutions. Those preparations described in the literature as "myosin" which contain considerable amounts of actomyosin, as deduced from the way of preparation, will be designated as actomyosin (e.g. the "myosin" of the era preceding the discovery of actin). The myosin α electrophoretic component of Dubuisson (1946a, b) corresponds to actomyosin.

Tropomyosin denotes tropomyosin, the soluble tropomyosin of Bailey (1946, 1957), and the tropomyosin B of Kominz *et al.* (1957c).

Paramyosin denotes paramyosin (Hall *et al.*, 1945), the insoluble tropomyosin of Bailey (1957c), and the tropomyosin A of Kominz *et al.* (1957c).

Since 1950, a number of books (Mommaerts, 1950; Szent-Györgyi, 1951, 1953; Dubuisson, 1954) and reviews (Weber and Portzehl, 1952, 1954; Bailey, 1954; Mommaerts, 1954; Hamoir, 1955a; Hanson and

Huxley, 1955; Hasselbach and A. Weber, 1955; Morales *et al.*, 1955; A. G. Szent-Györgyi, 1955; Buchthal *et al.*, 1956; Feigen, 1956; Perry, 1956; Weber, 1957; Gelfan, 1958) have appeared which discuss the properties of the fibrous proteins of muscle, their contribution to the filamentous organization of the myofibril, and their participation in the various activities of the muscle cell. These works represent the different aspects and points of view of the various schools working on these lines.

II. CHARACTERIZATION AND REACTIONS OF THE MYOFIBRILLAR PROTEINS

A. Myosin

1. *General Properties and Isolation*

Schramm and Weber (1942), in the first ultracentrifugal studies of "myosin" solutions, observed that such solutions contain a slow sedimenting component, $S_{20} = 6$, and faster sedimenting components, $S_{20} = 20$ to 36. These components were separated by differential centrifugation. At the same time, Banga and Szent-Györgyi (1941) found great differences in the viscosity of the salt extract, depending on the time of extraction. Short extraction yielded myosin which had a relatively low viscosity, and the viscosity of the solution was little influenced by the addition of ATP. If the muscle mince was extracted overnight with the same salt solutions, the extract had a high viscosity, and the viscosity was greatly lowered on addition of ATP. They deduced that during prolonged extraction, a second protein was extracted which complexed with myosin, and that the "myosin" of the older literature represented such a complex of two proteins. Actin, the protein extracted along with myosin, was isolated and characterized by Straub (1942, 1943), and the way was opened to prepare and characterize actin-free myosin.

Myosin constitutes a major fraction of the myofibrillar proteins and has been found in all muscles studied. It is readily soluble at ionic strengths higher than 0.3. The solutions are fairly viscous as a result of the considerable asymmetry of the particles. The dependence of the viscosity on concentration up to 1–2% protein concentration follows the Arrhenius law: $\log \eta \text{ rel} = K \times C$, and remains normal at moderate shear rates (Weber, 1950). The viscosity of an actin-free myosin preparation is not changed by addition of ATP or pyrophosphate and Mg^{++} ions. Such a preparation shows little flow birefringence. A com-

parison of the viscosity before and after the addition of ATP is the simplest and a sensitive method of detecting the presence of actin.

If the ionic strength is decreased below 0.2, the protein precipitates. The ionic strength at which myosin dissolves depends on pH and on the presence of ions like Mg^{++}, I^-, or ATP.

At neutral pH, precipitation is complete in 0.025 to 0.05 *M* potassium chloride solutions. If a myosin preparation which is dissolved in 0.6 *M* potassium chloride is diluted in the cold by 12 volumes water under vigorous stirring, the precipitate gives a silky appearance, and in the microscope, small needle-shaped crystals are seen, "crystalline myosin" (Szent-Györgyi, 1943). The crystals are composed of regularly associated fibrils, as shown by electron microscopic studies (Jakus and Hall, 1947; Snellman and Erdös, 1948a, b). The more amorphous precipitates still consist of fibrils. When ions are removed by dialysis, myosin even at very low protein concentrations forms a gel which consists of a network of filaments built of a regular association of molecules.

Most of the procedures for the preparation of myosin are based on extraction and repeated dissolution of the protein in solutions of high potassium chloride concentration, followed by repeated precipitation at lower potassium chloride concentration. By taking proper care in keeping the temperature low and avoiding contamination with heavy metals, fairly monodisperse preparations can be obtained.

The main impurities are actin, ribonucleic acid (Mihályi *et al.*, 1957), and lipids, carried along with the myosin precipitate.

For the removal of actin, several procedures have been developed. These are based on differences in solubility between myosin and actomyosin in potassium chloride or in ammonium sulfate. Actomyosin can be removed by differential precipitation at slightly alkaline pH and low ionic strength (Szent-Györgyi, 1943), at neutral pH and ionic strength of 0.3 (Portzehl *et al.*, 1950) in the absence of ATP, or at slightly alkaline pH and ionic strength 0.15 in presence of ATP (Spicer and Gergely, 1951).

Actomyosin was observed to precipitate irreversibly if dialyzed against water, and can be separated from myosin (Mommaerts and Parrish, 1951). Since myosin precipitates at higher ammonium sulfate saturation than actomyosin (Dubuisson, 1946a, b), salting out in ammonium sulfate has been used to obtain an electrophoretically homogenous preparation (Tsao, 1953a).

These methods are not suitable for removal of large amounts of actin

contamination. Actin combines with several times its weights of myosin and solubility differences, in most cases, are not sharp enough to permit high yields. It is preferable if the first extract contains as little actin as possible. This is the basis of Szent-Györgyi's original procedure, where pH, ionic strength, degree of homogenization of the muscle, and the time of extraction, are carefully controlled. His myosin A contains about 1% or less actin by weight, and about 0.5% ribonucleic acid. This method is suitable for fresh rabbit, chicken, or turkey breast muscles, but even with these muscles, the same procedure will extract more actin with increased comminution of the homogenate in the Waring Blendor (Hasselbach and Schneider, 1951).

From other muscles, it may not be possible to extract myosin without a considerable amount of actin.

Actin can be removed from actomyosin by employing differential centrifugation in the presence of sufficient amounts of ATP to last throughout the time of centrifugation (A. Weber, 1956). The sedimentation rate of actin is about 10 times faster than that of myosin. ATP dissociates the actomyosin complex and the actin-free myosin may be recovered from the supernatant solution. This procedure was succesfully applied for the preparation of actin-free myosin from Pecten maximus (Ruegg, 1957). An alternative method is to treat actomyosin with 0.6 *M* potassium iodide in the absence of ATP (A. G. Szent-Györgyi, 1951a). Under these conditions, potassium iodide denatures actin, and myosin may be recovered by reprecipitation in potassium chloride.

Certain invertebrate muscles contain paramyosin, a protein which has a somewhat similar solubility to myosin. With these muscles, special care has to be taken to separate the two proteins.

Ribonucleic acid accounts for about half of the phosphorus found in myosin preparations obtained from rabbit, and can be removed by ribonuclease digestion. It is not necessary for the various activities of myosin; ATPase activity and combination with actin are fully retained after RNAase treatment (Mihályi *et al.*, 1957).

2. *Dimensions*

There are a number of difficulties in determining the size and shape of myosin. The molecule is very asymmetric as indicated by its intrinsic viscosity of 2.0 (Portzehl *et al.*, 1950). Myosin is heat sensitive and aggregates readily at moderate temperatures. It has a great tendency to surface denaturation.

TABLE I
SOME CONSTANTS OF FIBROUS MUSCLE PROTEINS

Protein	$S^{\circ}_{20} \times 10^{13}$	$D^{\circ}_{20} \times 10^{7}$	Intrinsic viscosity	Partial specific volume	Length (A.)	Width (A.)
Myosin	7.2[1,2] 7.1[3] 6.7[4,5] 6.25[6] 6.1[7,8]	0.87[9] 1.05[5]	2.0[3] 1.8[10] 2.0[11] (cod)	0.728[5]	2,200–2,400[9] 1,500[12] 1,750[12] 1,600[14]	2–22[4,9]
G-actin	2.5–4.0[20] 3.7[3] 2.8–3.1[21]	2.5[20]	0.21[22] 0.075[11] (cod)		290[22]	24[22]
F-actin	50[2,26] 65[3]					
Tropomyosin	2.6[27] (c = 0.6%) 2.85[19] (carp) 3.2[28] (squid) 3.0[29,30] (rabbit skeletal and uterine, bovine bladder, *Venus*, *Loligo*, *Homarus*)	2.4[27] (c = 0.6%	0.52[31]	0.71[27]	400[31]	15[32]
Paramyosin	3.0[30] (*Lumbricus*, *Arenicola*, *Venus*) 3.1[36] (*Pinna*)	2.21[36]	2.0[37] (in urea) 2.56[37] (1 *M* KCl)	0.736[36]	1,400[36]	

[1] Snellman and Erdös, 1948a.
[2] Johnson and Landolt, 1950
[3] Portzehl *et al*,. 1950.
[4] Mommaerts and Parrish, 1951
[5] Parrish and Mommaerts, 1954
[6] von Hippel *et al* , 1958
[7] Laki *et al* , 1952.
[8] Miller *et al* , 1952
[9] Portzehl, 1950.
[10] Weber and Kerekjárto, 1952.
[11] Connell, 1954.
[12] Mommaerts, 1951b.
[13] Steiner *et al* , 1952.
[14] Holtzer and Lowey, 1956
[15] Laki and Carroll, 1955.
[16] Gergely and Kobler, 1957.
[17] Mommaerts and Aldrich, 1958.
[18] Dubuisson, 1950a

TABLE 1 (*continued*)

SOME CONSTANTS OF FIBROUS MUSCLE PROTEINS

Calculated from	Particle weight	Calculated from	Isoelectric point	Electrophoretic Conditions: Ascending Mobility (× = 10^5) (cm²/v/sec)	pH	Ionic Strength
S°_{20} and M.W. light scattering	850,000[20]	S°_{20} and D°_{20}	5.4[13]	2.9[10]	7.3	0.4
	840,000[29]	osmotic pressure	5.3[11] (cod)	2.8[19] (carp)	7.1	0.35
	500,000[13]	S°_{20} and D°_{20}		2.9[11] (cod)	7.4	0.4
	530,000[14]	light scattering				
	500,000[16]	light scattering				
	400,000[8]	S°_{20} and D°_{20}				
	420,000[8]	Archibald				
	390,000[17]	Archibald				
Viscosity and M.W.	150,000[20]	S°_{20} and D°_{20}	5.05[24]	4.6	7.4	0.4
	120,000[23]	light scattering	4.7–4.8[11] (cod)	3.9[11] (cod)	7.4	0.4
	80,000[13]	light scattering				
	57,000[21]	light scattering				
	70,000[22]	fluorescent polarization				
	74,000[22]	osmotic pressure				
	140,000[22]	fluorescent polarization	4.85[24]	6.3[23]	7.4	0.4
	1,500,000[13]	light scattering	4.7–4.8[11] (cod)	6.5[11] (cod)	7.4	0.4
	3,000,000[14]	light scattering				
Viscosity and M.W.	53,000[30] (urea)	osmotic pressure	5.1[33]	5.6[34]	7.4	0.4
	55,000[33]	light scattering	5.0[19] (carp)	7.1[30]	7.1	0.2
			5.4[28] (squid)	4.1[28] (squid)	7.4	0.35
				6.4[28] (squid)	7.4	0.15
				4.7[19] (carp)	7.1	0.35
				7.1[19] (carp)	7.3	0.15
				6.7[30] (*Venus*)	7.1	0.2
				6.9[30] (*Busycon*)	7.5	0.2
				9–11[35] (Bovine, rabbit, rat)	6.5	0.1
Light scattering	131,000[36]	S°_{20} and D°_{20}		4.4[30] (*Lumbricus*)	7.1	0.2
	137,000[36]	light scattering		4.0[30] (*Venus*)	7.1	0.2
				4.5[30] (*Busycon*)	7.5	0.2

[19] Hamoir, 1955a.
[20] Snellman *et al.*, 1947.
[21] Mommaerts, 1952a
[22] Tsao, 1953b.
[23] Johnson and Landolt, 1951.
[24] Debain, 1956
[25] Dubuisson, 1950b.
[26] Mommaerts, 1952b.
[27] Bailey *et al.*, 1948.
[28] Yoshimura, 1955.
[29] Kominz *et al.*, 1957c.
[30] Kominz *et al.*, 1957b.
[31] Tsao *et al.*, 1951.
[32] Doty and Sanders, quoted by Bailey, 1954
[33] Bailey, 1948.
[34] Dubuisson, 1950c.
[35] Tsao *et al.*, 1956.
[36] Kay, 1958.
[37] Bailey, 1956.

There is a considerable variation among the sedimentation constants obtained for myosin (cf. Table I). It was reported that the constant may depend on temperature and possibly on the centrifugal force (Parrish and Mommaerts, 1954).

For a number of years, the molecular weight of myosin appeared to be around 850,000. This was obtained by sedimentation-diffusion measurements performed at room temperature (Portzehl *et al.*, 1950). Osmotic pressure measurements carried out at 0° C. yielded a value of 840,000 (Portzehl, 1950). The observation that the sedimentation constants reported appeared to be lower and that the diffusion constants appeared to be higher in measurements performed in the cold, as compared with experiments carried out at room temperatures, led to the proposal that the minimum molecular weight of myosin was less than 500,000 (Laki and Carroll, 1955). A minimum molecular weight of 423,000 was derived, based on the molecular weights of the meromyosins. In this case, the 850,000 value would be the weight of a stable myosin dimer.

Molecular weights in the range of 400,000 to 500,000 were obtained by light scattering measurements (Holtzer and Lowey, 1956; Gergely and Kohler, 1957), and with the sedimentation-equilibrium technique of Archibald (Mommaerts and Aldrich, 1958; von Hippel *et al.*, 1958). Light scattering indicated a length of 1600 A. and the curves could be best fitted by assuming a rigid rodlike shape of the molecule (Holtzer and Lowey, 1956).

Flow birefringence studies indicate a polydispersity of the length of the particles in myosin preparations, the degree of which depends on the method of preparation, pH, the kind of buffer used, and protein concentration (Joly *et al.*, 1955). A discontinuous series of sizes calculated could be explained by assuming an equilibrium between the various degrees of association.

In conclusion, there is an indication that myosin in solution may exist both in a fairly stable monomer and in a dimer form. The formation of the dimer is facilitated by a short exposure to moderate temperatures (Holtzer, 1956). Dimerization does not seem to involve a loss in capacity to combine with actin or in ATPase activity. Higher temperatures cause further aggregation, resulting in the formation of particles of varying sizes. During this process, the specific activities of myosin may disappear. Sensitivity to temperature may vary considerably from species to species. The spontaneous aggregation has been

observed to take place much faster in fish and frog than in rabbit myosin, with simultaneous loss of ATPase activity (Connell, 1954; Hamoir, 1955b).

3. *Interaction with Ions*

It is an empirical observation that *in vitro* contraction of actomyosin takes place only under conditions where the protein is precipitated. The ATPase activity of myosin is influenced greatly by the presence of various ions. The understanding of the interaction of myosin with different ions is of great importance.

An isoionic point of myosin of pH 5.74 was obtained using Sorensen's technique, by determining the pH at which addition of KCl does not change the pH of a salt-free myosin solution (Mihályi, 1950). Electrophoretic mobility measurements (Erdös and Snellman, 1948) indicate an isoelectric point of 5.4 in potassium chloride solutions. The lack of shift in pH when sodium or potassium chloride is added to an isoionic myosin indicates that at this pH there is no preferential binding of these ions and that equivalent amounts of K^+, Na^+, and Cl^- are bound by myosin. Below the isoionic point, the binding of halides follow the Hofmeister series, $F^- > I^- > Br^- > Cl^-$, and there is a strong binding of SCN^- (Ghosh and Mihályi, 1952).

Studies employing equilibrium dialysis (Nanninga, 1957a) led to the conclusion that potassium ions were bound in some degree by myosin even at its isoelectric point. At more alkaline pH values, the binding of K^+ ions increases and is equivalent to the dissociated H^+ ions at 0.05 *M* KCl concentration (Nanninga, 1957). These conclusions are in agreement with the results of the cataphoretic mobility measurements of myosin at both sides of the wide precipitation zones, at different pH values and salt concentrations, from which it was concluded that the precipitation is an isoelectric one (Sarkar, 1950). Thus the net charge of a precipitated myosin is close to zero.

Extensive study of the sodium and potassium binding of myosin and actomyosin, using permselective membranes (Lewis and Saroff, 1957), showed a considerable binding of potassium and sodium, provided myosin was not exposed to higher temperatures (27°C). Heat-denatured myosin did not bind these ions. Sodium was bound more strongly than potassium. Addition of actin to myosin decreased the binding of these ions, and correspondingly actomyosin (myosin B, 5 hours extract) binds a smaller number of K^+ ions. From the pH dependence, the

authors deduced the presence of two kinds of binding sites (n_1, n_2) with $n_1 = 15$ and $n_2 = 35$. A comparison with the titration curves indicated that these sites were related to imidazol and amino groups which may modify the binding on the carboxyl groups. An analysis of the data led to the suggestion that K^+ and Na^+ were bound by chelate formation between carboxyl—alkalimetal—imidazol and carboxyl—alkalimetal-amino groups. Such a chelation mechanism would explain the difference in sodium and potassium binding and the observation that a configuration characteristic to the native protein is required for the binding of these ions (Saroff, 1957).

In the presence of magnesium or calcium ions, the electrophoretic mobility of myosin is greatly altered, and the isoelectric point is shifted to the alkaline direction. In 0.1 *M* $CaCl_2$, the isoelectric point of myosin is at pH 9.6 (Erdös and Snellman, 1948). The strong preferential binding of calcium or magnesium over chloride ion is shown by the shift of pH of isoionic myosin on addition of Ca^{++} and Mg^{++} (Ghosh and Mihályi, 1952). There is little difference between the binding of the two ions.

Equilibrium dialysis studies do not indicate differences between the two meromyosins as far as magnesium, calcium, and chloride ion binding is concerned (Nanninga, 1957). In contrast, at pH 8.2, light meromyosin (LMM) became positively charged and heavy meromyosin (HMM) remained negatively charged in presence of 0.05 *M* $CaCl_2$ (Erdös, 1955). Further studies are needed to resolve these differences.

The interaction of ATP with myosin is of particular interest. Unfortunately, the direct determination of ATP binding is difficult, since ATP is hydrolyzed by myosin and there is no obvious change in the absorption spectrum of ATP or myosin during their interaction. The association constants were deduced in an indirect fashion from the kinetics of ATPase activity (Ouellet *et al.*, 1952), and from the amount of ATP required to cause the dissociation of actin from myosin (Mommaerts and Hanson, 1956; Gergely and Kohler, 1957). These studies indicate a strong binding of ATP to myosin at sites of the enzymatic centers and at sites of interaction with actin. A more direct method to study binding of ATP by myosin is clearly desirable.

4. *Enzymatic Activity*

a. Mechanism of Hydrolysis of ATP. The importance of Engelhardt and Ljubimova's (1939) discovery that myosin acts as an adenosinetriphos-

phatase was recognized immediately. It meant that the protein intimately participating in contraction may have a direct regulatory activity on the rate of energy liberation; thus, this reaction may serve as a key for the understanding of mechanochemical coupling.

It is obvious that the hydrolytic step itself is not a prerequisite of energy transfer. For that matter, metabolic group transfer reactions are usually not accompanied by hydrolysis, which must come after such a transfer to the protein system has taken place, otherwise the energy would be wasted as heat. Still, the ATPase property of myosin may serve as a sign of its ability to be energized by ATP, even if the steps preceding hydrolysis are not clear at the moment. It is important, in this regard, that a modified myosin preparation, in which ATPase activity is abolished, is not contractile in presence of actin and ATP.

The mechanism of the ATPase action has been greatly clarified by recent isotope studies. The point of hydrolytic cleavage was determined by performing the hydrolytic reaction in the presence of O^{18} (Koshland *et al.*, 1954). Since all the O^{18} was recovered in the phosphate and none in the ADP, the bond hydrolyzed by myosin is the linkage between the last phosphorus and oxygen atom. The excess of O^{18} in the phosphate after hydrolysis by actomyosin at pH 9.0, with calcium as activator and in 0.1 *M* KCl, agrees with the theoretical amounts expected from a nonenzymatic hydrolysis, corresponding in amounts to the labeling of one of the four oxygen atoms of phosphate. There was no exchange between ADP^{32} or $KH_2P^{32}O_4$ and ATP in presence of myosin or actomyosin, and no O^{18} was taken up from the medium by unhydrolyzed ATP or by inorganic phosphate. The results are thus compatible with a single displacement mechanism by water during which no covalent bond was formed as a result of P-O cleavage. A double displacement mechanism with a phosphorylated intermediate could occur only if it was of a transient nature and was immediately hydrolyzed.

A different result was obtained when strips of washed lobster muscle were used in about 0.05 *M* KCl in absence of calcium (Koshland and Clarke, 1953). On each phosphate group hydrolyzed the presence of about three O^{18} atoms was observed and a slow exchange took place between inorganic phosphate and the medium. This apparent contradiction was resolved recently by the realization of the role different ions may play in the exchange reaction (Levy and Koshland, 1958). In presence of calcium only one O^{18} atom was found on each phosphate hydrolyzed by myosin or actomyosin (prepared by mixing purified

myosin and purified actin). When calcium was replaced by magnesium at low ionic strength, the phosphate group liberated by myosin contained excess of O^{18} corresponding in amounts to the labeling of about three of the four oxygen atoms of phosphate; the excess of O^{18} on the phosphate group liberated by actomyosin corresponded to the labeling of about two of the four oxygen atoms of phosphate. It is important that actomyosin, under these conditions, hydrolyzed ATP more than 10 times faster than did myosin free from actin. Actin alone was inactive in splitting phosphate from ATP or in the exchange reaction. The fact that the incorporation of O^{18} atoms did not decrease to the value theoretically expected from simple hydrolysis in the experiments in which actomyosin was used, and the hydrolysis rate was greatly increased, is a strong indication for the specificity of myosin in this reaction.

The importance of the results of these studies is obvious, since they strongly suggest that preceding hydrolysis myosin becomes phosphorylated. The nature of the phosphorylated intermediate is not yet known, except that it probably is of a transient nature and is hydrolyzed rapidly. The transient nature may be one explanation of the negative results of the exchange experiments using phosphate labeled ADP or inorganic phosphate. Further studies along these lines and its correlation with contractility may have far reaching importance in our understanding of the energization step.

A rapid incorporation of phosphate labeled ADP into ATP was reported using well washed myofibrils both before and after extraction of myosin, and using repeatedly reprecipitated actomyosin (myosin B) in presence of magnesium but not in presence of calcium. Purified actin or myosin or "synthetic" actomyosin did not catalyze such an exchange. It was suggested that the exchange was facilitated by the actin component of muscle (Ulbrecht and Ulbrecht, 1957). Though enzymes like myokinase or nucleotide phosphokinase were excluded from the reaction (Ulbrecht *et al.*, 1957) it should be noted that the preparation method used for both the myofibrils and actomyosin were not necessarily optimal for the removal of granules capable of catalyzing such an exchange reaction and their contribution has not been excluded.

The water participating in the hydrolytic step is not free water in solution, but is associated with the protein or ATP (Koshland and Herr, 1957). This was shown in experiments where C^{14}-labeled methanol was used in concentrations low enough (10^{-3}–$10^{-1}M$) so as not to influence the properties of the solvents and the enzymatic properties

of the protein. The relative reactivity of water and methanol was obtained from the bimolecular rate constants of hydrolysis and methanolysis by measuring methyl phosphate and inorganic phosphate. In nonenzymatic reactions, there was little preference for water over methanol, using ATP and a wide range of phosphate compounds. The relative nonenzymatic reactivity of water and methanol was constant and the ratio of the bimolecular rate constants stayed within the range of 0.4 to 2.5 for the various phosphate compounds. Quite different values were obtained for the enzymatic reaction with myosin. No measurable amounts of methyl phosphate could be found. The ratio of the rate constants of hydrolysis and methanolysis was greater than 300 and probably higher than 1,000. Thus, water does not freely enter from the solution in the hydrolytic step. It may come either from a specific site of the protein on which water is adsorbed directly, or there may be a site for hydrated ATP on which water is adsorbed only in conjunction with the phosphate compound. A third and least likely possibility is that the site for ATP can be approached only by water, but not by methanol, because of steric considerations.

b. Specificity and Activators. Myosin ATPase was extensively studied and there is a large literature on the effect of various activators and modifiers. ATPase activity is one of the most sensitive properties of myosin. Inactivation of the enzyme occurs after exposure to moderate temperatures and slight acidification. In many cases, enzymatic activity is lost before drastic changes in solubility of myosin take place. High activity, with Q_P values of around 15,000, was observed on preparations used very soon after extraction (Mommaerts and Green, 1954). The correlation between aggregation of myosin during standing and inactivation of ATPase activity has not been thoroughly studied.

In presence of Ca^{++} ions, there are two pH optima, one at pH 6.4, the other at more alkaline pH values. The alkaline pH optimum depends on temperature and duration of the experiments, and is influenced by the ease of the denaturation of myosin at various pH's and temperatures. In experiments of short duration at 27°C. the optimum was around pH 10.0, as opposed to the pH 9.2 value found at 37°C. (Mommaerts and Green, 1954). The alkaline pH optimum may be explained by the participation of OH-ions, instead of water, in the splitting reaction (Ouellet *et al.*, 1952).

The activation of myosin ATPase by ions is of a complex nature and

there is a mutual interaction of a number of parameters. If several ions are present simultaneously, the concentrations required for optimal activity differ from the optimal concentrations when the same ions are used separately. Some of the ions which are activators, if present alone, become inhibitors in the presence of other ions. Ca^{++} ions are potent activators; K^+ activates also, but to a lesser extent. Na^+ decreases the activity of K^+ activitated ATPase, but the concentrations of Na^+ required are considerably higher than the Na^+ concentration in muscle (Mommaerts and Green, 1954). Mg^{++} ions alone inhibit enzymatic hydrolysis. The most obvious effect is the strong inhibition of Ca^{++} activitated myosin ATPase in presence of Mg^{++}.

In presence of modifiers, especially actin, which in itself is not an ATPase, the activating and inhibiting effect of ions may change qualitatively. Thus Mg^{++}, which inhibits myosin ATPase, will activate actomyosin ATPase at neutral pH (Banga and Szent-Györgyi, 1943).

The enzymatic specificity of myosin is fairly great toward the phosphate end of the substrate, and only the last phosphate of the triphosphorylated nucleotides is hydrolyzed. There is a broad specificity toward the nucleotide end. ITP, UTP, CTP, GTP, and acetyl-ATP are all readily hydrolyzed by myosin (Kleinzeller, 1942; Deutsch and Bergkvist, 1955; Bergkvist and Deutsch, 1954; Blum, 1955; Hasselbach, 1956). Inorganic triphosphate is hydrolyzed also, though at a slower rate (Friess and Morales, 1955). Both ADP and inorganic pyrophosphate have a considerable inhibitory action.

The rate of hydrolysis of the various triphosphorylated nucleotides differs. It depends on the type of ions used, and changes depending on whether myosin or actomyosin is used as enzyme. In general, the nucleotides having an –OH group at the 6-position are split faster by myosin than are nucleotides having an NH_2 group at the same position. With actomyosin, the situation is reversed and the enzyme will split nucleotides with an NH_2 group in the 6-position faster than nucleotides with an –OH group in the 6-position (Hasselbach, 1957a). It is evident that a multiple binding of the substrate via the phosphate chain and the nucleotide base is involved. Some of the variations may be explained by differences in binding. Thus, the association constant derived from Michaelis-Menten kinetic considerations increases as the hydrolysis rate decreases, indicating that the variations may be partly due to differences in the desorption of the product from the enzyme (Blum, 1955).

Changes in bind g or orientation of the nucleotides may be a partial

explanation of the strong activating effect of chelating agents. Depending on the ions present, ethylenediaminetetraacetate may be a powerful activator of the hydrolysis of ATP, and to a somewhat lesser extent of CTP (Friess, 1954; Bowen and Kerwin, 1954). The splitting of nucleotides with an –OH group in the 6-position is not increased or decreased by this chelating agent (Hasselbach, 1957a). The activating effect of EDTA depends on the presence of monovalent cations. Na^+ and Li^+ have no effect, with K^+ the activation is considerable, and NH_4^+ is the most potent activator (Kielley *et al.*, 1956).

It is of particular interest that EDTA does not act by simply removing metal contaminations which inhibit ATPase activity. Addition of EDTA has the same effect on a myosin which was prepared and repeatedly reprecipitated in presence of EDTA as on an untreated preparation (Friess *et al.*, 1954; Bowen and Kerwin, 1954). Other agents which chelate Mg^{++} and Ca^{++} ions have an action similar to that of EDTA, while agents which do not chelate alkaline earth metals have little effect on myosin ATPase (Bowen and Kerwin, 1954). The possibility arises that EDTA activation is due to the chelation with strongly bound Mg^{++} or Ca^{++}. These ions may be involved in the binding of the nucleotide base or in its fixation in an orientation favorable to the hydrolytic process. It would appear that the NH_2 group at the 6-position is of particular importance in this reaction.

The importance of the –SH groups of myosin for ATPase activity has been early recognized. Treatment with *p*-chloromercuribenzoate (PCMB) abolishes enzymatic activity (Singer and Barron, 1944). Both ATPase activity and the capacity of myosin to combine with actin decrease in a parallel fashion (Bailey and Perry, 1947). However, not all the –SH groups of myosin are necessary for the hydrolysis of ATP. Partial reaction with PCMB or *N*-ethylmaleimide (NEM) increases the rate of ATP hydrolysis up to the point when about half of the –SH groups of myosin have reacted (Kielley and Bradley, 1956). Phenylmercuric acetate and 2, 4-dinitrophenol (DNP) activate at low concentration and inactivate at higher, possibly through the same mechanism (Greville and Needham, 1955; Chappell and Perry, 1955). It is important that activation by partial abolition of –SH groups is obtained only if Ca^{++} is used as activator. In presence of EDTA, ATPase activity decreases even at low concentrations of PCMB or NEM (Kielley and Bradley, 1956). Only the nucleotides with NH_2 groups in the 6-position and not those with –OH groups in the 6-position are activated with

–SH reagents (Greville and Reich, 1956). It is possible that some of the –SH groups reduce the rate of reaction by "orienting" the base in a position not favorable for hydrolysis.

Activation of myosin ATPase by EDTA, PCMB, zinc ions, and DNP occurs only at room temperature (25°C.), and the same reagents will inhibit at 0°C. even at low concentrations. These results led to the proposal that activation requires the previous aggregation of myosin by exposure to room temperature, and that it consists of the reversal of this aggregation. The aggregation accordingly would modify the active centers by possibly involving –SH groups (Gilmour and Griffiths, 1957).

The parameters influencing the ATPase activity of myosin are numerous, and it is difficult to account for all of them in a unified and simple fashion. Physiologically, the most important is obviously actin and the effect of ions. A comparison of the hydrolysis of the various nucleotide triphosphates and the effect of chelating agents and –SH interactions gives information about some of the parameters having a role in the binding of substrate to enzyme. The correlation of these effects with the formation of the phosphorylated intermediate of Koshland will be of great interest. Such studies may help to separate the steps necessary for the successful energization of the molecule, preceding the otherwise energetically wasteful hydrolysis.

5. *Components of Myosin*

Myosin can be dissociated by a variety of agents. These include reagents which do not break peptide bonds and proteolytic enzymes. The products into which myosin is broken up differ from each other. There are indications that myosin is a compound protein built of nonequivalent components. ATPase activity and ability to combine with actin are lost when the myosin is dissociated with reagents breaking secondary linkages only, but these properties of myosin are fully retained when the components are obtained after controlled incubation with proteolytic enzymes.

a. Components Obtained by Breaking Secondary Linkages. In urea, the viscosity of myosin is decreased considerably, and the molecule depolymerizes into smaller units (Weber and Stover, 1933; Edsall *et al.*, 1939). After short treatment with urea, the viscosity change appears to be reversible, and for depolymerization, several days of incubation are

required (Snellman and Erdös, 1948b). The reaction was studied in detail after very prolonged urea treatment lasting for several months (Tsao, 1953a). A relatively small and a relatively large molecular weight component were recovered after such treatment. The units of an average molecular weight around 16,000 comprised about 8% of the myosin by weight, showed an unusual resistance to heat treatment, and was soluble at fairly high ethanol concentrations. The rest of the molecule had a high molecular weight after removal of urea, but depolymerized instantaneously at pH 10.7, yielding particles of an average molecular weight of 170,000. No N-terminal group was found on the larger units, while the smaller units contained several different amino acid residues whose sum was equivalent to one N-terminal group per 16,000 units of molecular weight. It was proposed that myosin was composed of an equal number of larger cyclic units and smaller open chain polypeptides. The number of such units naturally depends on the molecular weight of myosin. In a myosin monomer, their number would be two or three (Table II).

Even after short urea treatment, all the specific activities of myosin are irreversibly lost, and the preparation does not combine with actin (Snellman and Erdös, 1948b). The small molecular weight components can appear even after short urea treatment (Middlebrook and A. G. Szent-Györgyi, 1958), or after incubation with NaHCO, at pH 10 (Kominz *et al.*, 1958). Denaturation of myosin by these two reagents yields components of molecular weight about 30,000, having apparently identical amino acid composition and properties, and comprising about 10% of the myosin by weight. If myosin is subjected to moderate temperatures (53°C.), the main part of the molecule aggregates, and several fragments soluble at low ionic strength are detached (Locker, 1956). Some of these may bear a relationship to the small units obtained after urea or alkali treatment.

Thus, it would appear from these studies that the myosin molecule is made up from several large components of an average molecular weight around 200,000. The smaller molecular weight units are attached in such a fashion that they are released when the molecule is denatured. The loss of specific activities and the drastic changes in the solubility properties, after the use of denaturing agents, makes it difficult to determine how far the larger subunits are equivalent, and how the various components contribute to the different functions of myosin.

TABLE II

SOME CONSTANTS OF COMPONENTS OF MYOSIN

Component	$S^\circ_{20} \times 10^{13}$	$D^W_{20} \times 10^7$	Intrinsic viscosity	Partial spec. vol.	Length (A.)	Width (A.)	Calc. from	Particle weight	Calculated from
HMM	6.96 (tr)[b] 7.2 (ch)[c]	2.91 (tr)	0.28–0.36 (tr) 0.25–0.40 (ch)	0.748[a] (tr) 0.74[a] (ch)	430	30	f/f_0	232,000[1]. (tr) 220,000[2]. (ch) 320,000[3]. (tr, ch)	$S^\circ_{20} + D^\circ_{20}$ S°_{20} + viscosity $S^\circ_{20} + D^\circ_{20}$, light scattering[3]
LMM	2.86 (tr) 2.8 (ch)	2.87 (tr)	1.0 (tr) 1.0 (ch)	0.748[a] (tr) 0.74[a] (ch)	550	16.6	f/f_0	96,000[1]. (tr) 100,000[2]. (ch) 140,000[3]. (tr, ch)	$S^\circ_{20} + D^\circ_{20}$ S°_{20} + viscosity $S^\circ_{20} + D^\circ_{20}$, light scattering
LMM Fr. 1 (tr)			1.0	0.748[a]				120,000[4]	Archibald
Protomyosin (tr)	0.82	17.4	0.065	0.748[a]				4,600[5]	$S^\circ_{20} + D^\circ_{20}$
Fraction A obtained after long urea treatment at pH 10.7			0.7	0.74[a]				165,000[6] 165,000[6]	fluorescent polarization osmotic pressure
Fraction B obtained after long urea treatment			0.2	0.74[a]				16,000[6] 14,000[6]	fluorescent polarization osmotic pressure

[a] Assumed value.
[b] (tr) Obtained after tryptic treatment.
[c] (ch) Obtained after chymotryptic treatment.

References cited in Table II
[1] A. G. Szent-Györgyi, (1953).
[2] Gergely *et al.*, (1955).
[3] Gergely *et al.* (1958).
[4] Cohen *et al.*, (1958).
[5] A. G. Szent-Györgyi and Borbiro (1956).
[6] Tsao (1953a).

b. Components Obtained with the Aid of Proteolytic Enzymes. Some of the proteolytic enzymes change the properties of myosin in a rather unique fashion. As first observed short incubation of a myosin solution with trypsin caused a considerable decrease in the viscosity of the solution, left the ATPase activity unimpaired, and concurrently the ATPase became soluble in water or in solutions of low ionic strength (Gergely, 1950; Perry, 1951). Subsequently, it was shown that trypsin action may be conveniently divided into two phases. The viscosity decrease occurs in the early phase of digestion, during which only a small amount of nonprotein nitrogen is split off (Mihályi, 1953). During this reaction, there is no decrease in the enzymatic activity of myosin, and it still combines with actin (Mihályi and A. G. Szent-Györgyi, 1953a). In the second phase of digestion, nonprotein nitrogen is liberated and the specific activities of myosin are lost. Early trypsin digestion results in the appearance of two components, one sedimenting slower, the other slightly faster than the intact myosin molecule (Mihályi and A. G. Szent-Györgyi, 1953b). The reaction appears to occur in an all-or-none fashion, i.e., within the resolution of the ultracentrifuge no accumulation of intermediary components can be observed, and the ratio of the two peaks remains constant during digestion. Since only the faster sedimenting component combines with actin, it could be separated from the slower sedimenting component by differential centrifugation, taking advantage of the high sedimentation rate of actin. The two fragments have a different solubility at low potassium chloride concentrations, and their salting-out curves differ in ammonium sulfate. Good separation was achieved, based on these solubility differences. The slower sedimenting component was called light meromyosin (LMM), and the faster sedimenting one, heavy meromyosin (HMM). LMM was obtained in needle-shaped crystals (A. G. Szent-Györgyi, 1953).

One of the most significant aspects of the meromyosins is that they fully retain some of the specific properties of myosin. HMM has the full ATPase activity of myosin and combines with the same amount of actin as the intact myosin molecule. It differs from myosin in that it is soluble in water or in low concentrations of potassium chloride and that it salts out at a higher ammonium sulfate saturation. LMM has no ATPase activity and does not combine with actin. It is similar to myosin in its solubility characteristics. LMM is precipitated in low concentrations of KCl, forms a gel in water, and salts out at the same ammonium sulfate concentrations as myosin. The properties of myosin are

thus segregated sharply between the meromyosins; this presents a useful tool for a differential study of the various reactions of myosin.

The meromyosins have a fairly large molecular weight. LMM has a molecular weight in the neighborhood of 100,000. The molecular weight reported for HMM ranged from 230,000 to 330,000 (A. G. Szent-Györgyi, 1953; Gergely *et al.*, 1958). In the earlier studies, it was proposed that there are two LMM units for each HMM unit in myosin. This calculation was based on a weight ratio of 0.57 HMM and 0.43 LMM. These values suggest a minimum molecular weight of 423,000 for the myosin monomer (A. G. Szent-Györgyi, 1953). The recently reported molecular weights for the meromyosins agree better with the proposal that there is one LMM for one HMM, in which case the myosin molecule is split into two fragments, the ratio of LMM to HMM being 0.27 to 0.73. The minimum molecular weight of the myosin monomer is 470,000 based on this proposal (Gergely *et al.*, 1958). It is difficult though at present to reconcile such a picture with the fractionation of LMM into at least two components of different properties but same molecular weight.

The LMM component of myosin was further fractionated by ethanol or acetone treatment. About 60% of an LMM preparation is not denatured by these solvents and redissolves in 0.6 *M* KCl after precipitation with three volumes of ethanol or acetone (Cohen and A. G. Szent-Gyorgyi, 1957). The component, called light meromyosin Fraction 1 (LMM, Fr. 1), has the same solubility properties, electron microscopic periodicity and small angle X-ray diffraction spacings as the intact LMM preparation. Its molecular weight determined by the Archibald technique is in the range of 120,000, a value close to that of the unfractionated LMM preparation (Cohen *et al.*, 1958). A fraction apparently identical with LMM, Fr. 1 was obtained if myosin was denatured first with ethanol and then digested with trypsin (Laki, 1957a).

In urea about 70% of the LMM depolymerizes into units having a molecular weight of 5,000 (protomyosins). LMM Fr. 1 also depolymerizes into such units. The depolymerized components are not equivalent and in electrophoretic field, the preparations show a number of peaks. The protomyosins are soluble in water, are fairly heat stable, and remain soluble at high ethanol concentrations. The depolymerization of LMM appears to be irreversible and removal of urea does not cause the reaggregation of these units (A. G. Szent-Gyorgyi and Borbiro, 1956).

The amino acid content of the two meromyosins differ considerably (Kominz *et al.*, 1954). The amino acid residues of the meromyosins taken in a ratio of 0.57 HMM and 0.43 LMM add up closely to the amino acid composition of myosin. Thus no material, different in its amino acid composition from myosin or the meromyosins, was lost in considerable amounts during digestion or the subsequent preparation. This is in agreement with the low values for nonprotein nitrogen liberated during the early phase of digestion (Mihályi, 1953).

An end-to-end association of the meromyosins is compatible with the fact that HMM sediments somewhat faster than myosin (Lauffer and A. G. Szent-Györgyi, 1955). A series of LMM-LMM-HMM fits the light scattering data of myosin better than a series of LMM-HMM-LMM (Holtzer and Rice, 1957).

During the early phase of digestion, there is little change in optical rotation, and the proportionate sum of the specific rotations of the meromyosins agree with the values of the myosin. This would indicate that there is little unfolding of helical regions during the process when myosin is broken down into its components (Cohen and A. G. Szent-Györgyi, 1957). This observation may be connected with the preferential splitting of the linkage holding the units together.

The linkage connecting the meromyosins is not unambiguously decided. Beside trypsin, a number of enzymes of different specificity requirements produce components that cannot be distinguished from the meromyosins as far as size, shape, and properties are concerned. These enzymes are chymotrypsin (Gergely *et al.*, 1955), subtilisin (Middlebrook, 1958), and snake venom esterase (Laki, 1958). The simplest explanation would be to assume a short polypeptide chain connecting the meromyosins which is broken at slightly different loci when trypsin or chymotrypsin acts. Still, C-terminal groups expected from the specificity requirements of trypsin or chymotrypsin are not found in a mole to mole ratio and no differences in the N-terminal and C-terminal groups of meromyosins are obtained after treatment with trypsin, chymotrypsin, or subtilisin (Middlebrook, 1958). Some differences in the end groups of the homologous fractions obtained after tryptic and chymotryptic digestion were reported in another study (Gergely *et al.*, 1958). There is a possibility that the meromyosins are not covalently linked, and the action of the proteolytic enzymes is to loosen the structure in such a way that the myosin molecule dissociates, yielding the meromyosins. If that is the situation, one would expect

that methods could be worked out to obtain meromyosins from muscle or from myosin, without the use of proteolytic enzymes.

The meromyosins appear to have a metabolic independence. Labeled amino acids are incorporated into the LMM portion of myosin at a considerably faster rate than into the HMM portion. If a rabbit is killed 3 hours after injection of C^{14}-labeled phenylalanine and the various muscle proteins are isolated in pure conditions, the labeling in LMM is considerably higher than in HMM (Velick, 1956). The estimated half-life period of LMM was about 20 days, that of HMM, about 80 days. Studying the incorporation of C^{14}-glycine several days after injection, the concentration of labeled glycine in LMM was considerably higher than in HMM, though turnover rates cannot be calculated from these long term experiments (Schapira *et al.*, 1956). These results suggest that the meromyosins are synthesized and broken down independently, and that they, or the components they may contain, are subunits in the biogenetic sense. The determination of the turnover ratios of a number of different amino acid residues are required to decide whether the synthesis of the meromyosins occurs from the same or different pools.

In conclusion, it is clear that myosin is built of at least two different components. It can be degraded with the aid of various denaturing agents under conditions where peptide bond fission is excluded. The myosin molecule can be fragmented by a number of proteolytic enzymes in a specific fashion, though in these cases, the mechanism of how proteolysis contributes to the formation of the meromyosins is not yet known. The correlation and the extent of overlap between the units obtained after urea treatment and the meromyosins has not yet been clarified. It may be significant, though, that the average molecular weight of the alkali depolymerized units (170,000) is not too much different from the average molecular weight of the meromyosins.

B. Actin

1. *General Properties and Preparative Aspects*

The observation that prolonged extraction of muscle with salt solutions extracts "myosin B" which has different properties and reacts differently with ATP than "myosin A" obtained after short extraction (Banga and Szent-Gyorgyi, 1941), paved the way to the isolation and characterization of actin by Straub (1942, 1943).

Actin is unique among the fibrous muscle proteins, in that in the pres-

ence of salts, it aggregates, and in the absence of ions, it dissociates into monomers (Straub, 1943). While actin is soluble in water or low concentrations of neutral salt solutions, these solutions do not extract it readily from the muscle. The circumstantial evidence that actin is present in muscle in the fibrous form (Perry, 1952; Biro and Nagy, 1955) may explain the difficulties encountered in its extraction. Agents which depolymerize actin, like potassium iodide, extract actin readily (A. G. Szent-Györgyi, 1951a; Bárány *et al.*, 1957).

The muscle residue remaining after partial removal of myosin may serve as starting material for extraction of actin free of myosin (Straub, 1942, 1943). The residue is then dried with acetone or some other nonpolar solvent. This procedure denatures the myosin still present, and may remove lipids possibly involved in keeping actin in the muscle structure. The dried muscle powder is extracted with distilled water at neutral or slightly alkaline pH. The extracted actin is in the globular form (G-actin). It can be converted into the fibrous form (F-actin) by the addition of 0.1 *M* KCl and traces of $MgCl_2$. Once it became clear that, in presence of ATP, actin can be reversibly depolymerized (Straub and Feuer, 1950), a number of methods could be worked out for its purification. Perhaps the simplest procedure is that which takes advantage of the high sedimentation rate of F-actin (Mommaerts, 1951a; 1952a). F-actin sediments and forms a pellet within a few hours at 100,000 × *g*, and can be redissolved as F-actin in 0.1 *M* KCl or as G-actin using as a medium ion-free water containing a low concentration of ATP. Redissolution can be greatly facilitated with the aid of a glass homogenizer. Using butanol as a drying agent, electrophoretically homogenous preparations were obtained without centrifugation (Tsao and Bailey, 1953), though electrophoretic homogeneity and activity, in terms of polymerizability and combination with myosin, do not necessarily go parallel.

2. *Dimensions*

Hydrodynamic measurements of actin are frought with difficulties. G-actin exists only in the absence of salts, or can be obtained by depolymerization of F-actin with high concentrations of strongly electronegative anions like I^- or SCN^-. It is likely, though, that such a depolymerization is not 100% efficient and this poses serious limitations in interpreting the measurements obtained for G-actin, especially as far as its shape is concerned. The difficulties in obtaining accurate data for

size and shape of polymers, which are in a not too well defined state of aggregation as F-actin, are obvious. The values for the molecular weights of the actin monomer ranges from 57,000 measured in absence of ions (Mommaerts, 1952) to 70,000 (Tsao, 1953b) in 0.6 *M* KI. A high value of 0.21 was reported for the intrinsic viscosity of the actin monomer (Tsao, 1953b). This result would indicate a considerable asymmetry for the actin monomer, but the uncertainties in the state of aggregation may serously limit such an interpretation. A higher molecular weight for F-actin is indicated by its high sedimentation constant ($>50 S$) (Portzehl *et al.*, 1950; Johnson and Landolt, 1951; Mommaerts, 1952b) and high viscosity. Light scattering data indicate a molecular weight of about $1.5–3 \times 10^6$ (Steiner *et al.*, 1952; Gergely and Kohler, 1957). Fluorescent polarization studies were interpreted in an entirely different manner (Tsao, 1953b). According to these measurements, when F-actin is formed, the molecular weight of the monomer only doubles, increasing to 140,000. To reconcile the sharp contradiction with the results of the measurements employing other techniques it is assumed that the kinetic units of F-actin are dimers which form a gel as a result of weak associations between the dimers. An alternative explanation is that there is a greater freedom of rotation between the dimers and that this is what is actually measured by fluorescent polarization (Bailey, 1954). It should be remembered that this is a new technique and caution is warranted in interpretation, especially on a system as difficult as actin. Clearly, further experiments are needed to ascertain the size and shape of actin, both in its fibrous and in its globular form.

3. *Actin-Actin Interaction*

a. Effect of Ions. If sodium chloride or potassium chloride is added to a G-actin solution, after a lag period, its viscosity rises, indicating the formation of F-actin. The polymerization is probably a regular association of actin monomers and, based on the small-angle X-ray diffraction studies, it was concluded that the association occurs with "atomic precision" (Astbury *et al.*, 1947). The length of the F-actin molecule is, however, indefinite.

Actin preparations contain strongly bound $MgCl_2$ and $CaCl_2$ (Feuer *et al.*, 1948; Hasselbach, 1957b). Their presence appears to be necessary for polymerization, which is inhibited if actin is treated with chelating agents like sodium hexametaphosphate or EDTA. This inhibition can be reversed by the addition of excess Mg^{++}, but not with monovalent ions.

There is a fairly complicated mutual interaction of ions in inducing polymerization (Feuer *et al.*, 1948). Sodium or potassium chloride is most effective at 0.1 *M* concentration. Magnesium or calcium ions induce polymerization at lower concentrations. In the presence of magnesium, the lag period characteristic of polymerization induced by monovalent cations is abolished. Calcium has an inhibitory action in the simultaneous presence of potassium or sodium.

b. Role of Nucleotides in Actin-Actin Interaction. Actin contains strongly bound adenine nucleotides which influence it in several ways. ATP has a protective action on G-actin, which loses its ability to polymerize once ATP is removed (Straub and Feuer, 1950). There is a change in the nucleotides bound to actin, closely connected with polymerization. Furthermore, the availability of nucleotides to various enzymes also depends on the polymerized or depolymerized state of actin.

Whenever actin is depolymerized by removal of ions or by high concentrations of potassium iodide, it is also inactivated, and will not repolymerize in the proper ionic milieu. Depolymerization is freely reversible, however, in the presence of ATP (Straub and Feuer, 1950). Inactivation appears to be a rapid process, and the ATP has to be present during depolymerization. Thus ATP is ineffective if added a few seconds after a depolymerizing agent like 0.6 *M* KI (A. G. Szent-Györgyi, 1951b). Interestingly, the presence of an ATP resynthesizing system like phosphocreatine with creatine kinase will render depolymerization reversible (Strohman, 1958). If the ATP bound to G-actin is dephosphorylated by potato apyrase (Straub and Feuer, 1950), by the hexokinase system (Laki *et al.*, 1950), or by HMM (Strohman, 1958), loss of polymerizibility of actin occurs.

There is an intimate association of the adenine nucleotides with the polymerization process. G-actin contains considerable amounts of ATP (Straub and Feuer, 1950; Laki *et al.*, 1950). During the conversion of G-actin to F-actin, the nature of the bound nucleotide changes, and the ATP tightly bound to G-actin is converted into ADP. While actin in itself has no ATPase activity, parallel with the polymerization process, inorganic phosphate is liberated (Straub and Feuer, 1950; Laki and Clark, 1951). G-actin can thus be looked upon as a complex of ATP-actin, F-actin as a complex of ADP-actin (Straub and Feuer, 1950). The conversion of ATP to ADP appears to be a stochiometric one, and 1 mole of nucleotide appears to be converted for each actin

monomer (Mommaerts, 1952c). The specificity of ATP has not been properly investigated yet and it is not known whether other nucleotides can replace it. During depolymerization, ATP is not resynthesized (A. G. Szent-Györgyi, 1951b).

The importance of these observations naturally is in the interdependence of the protein-protein interaction with the dephosphorylation process. The nature of the binding of the adenine nucleotides is not clear, although the participation of magnesium has been suggested (Tsao, 1953b). It appears that binding is stronger to F-actin than to G-actin; at least, ATP can be removed by dialysis fairly easily from G-actin, while little ADP is lost from F-actin by the same process.

Although dialysis or repeated centrifugations causes but little change in the ADP content of F-actin, the nucleotides are easily removed from both forms of actin after heat denaturation at the isoelectric point, or by precipitation with trichloroacetic acid or perchloric acid. It appears that the constellation of side chains in the native configuration facilitates the binding of nucleotides. It is clear that the reactions of the actin-bound nucleotides to various enzymes, like hexokinase, myokinase, etc., is poor as compared with nucleotides in free solution. Even in this respect, there is a difference between F-actin and G-actin. HMM can dephosphorylate the ATP bound to G-actin and the G-actin-HMM complex will liberate creatine from phosphocreatine in presence of creatine kinase, at a fairly high rate. If HMM is complexed with F-actin, the ADP of F-actin is not available for such a reaction sequence and creatine is accumulated only at a very slow rate (Strohman, 1958). The actin-actin bond formed during polymerization involves ADP in such a fashion that it will become refractory to various enzymes.

Polymerization of actin is accompanied by a change in the reactivity of the –SH groups of the protein. If the –SH groups of G-actin are fully reacted with organic mercurials or arsenic compounds, polymerization is inhibited (Kuschinsky and Turba, 1951). The reactivity of F-actin and G-actin is different toward the same reagent. Mersalyl reacts with 4 moles of –SH groups if added to G-actin (calculated for 57.000 g.), but only with 2 moles if added to F-actin. If after removal of excess mersalyl, the F-actin is placed in urea or guanidine, 2 moles of –SH groups reappear which were made unvailable to the reagent by the actin-actin bonding. Reaction of amino groups of actin with acetic anhydride or formaldehyde leads to inactivation of actin (Bárány, 1956). High concentrations of ATP inhibit the depolymerization of F-actin

induced by mersalyl, formaldehyde, or urea. These experiments led to the proposal that hydrogen bonding between –SH and NH_2 groups (possibly derived from ATP) may play a role in the actin-actin bonding (Bárány, 1956).

4. *Actin-Myosin Interaction*

When solutions of actin and myosin are brought together, a complex, actomyosin, is formed. It is characterized by a viscosity higher than that of the sum of the component proteins, by a high sedimentation constant, and by a high molecular weight. Light scattering studies indicate an average molecular weight in the order of 20×10^6 (Blum and Morales, 1953; Gergely, 1956). The solubility of myosin and actomyosin differs somewhat and the difference can be used in separating the two proteins. Thus actomyosin precipitates at higher KCl concentrations and lower ammonium sulfate saturations than myosin. Actin and myosin do not combine at any ratio and if one of the components is in excess, its presence can be detected in the ultracentrifuge (Weber, 1950). The stoichiometry of the combination is around 1 g. of actin to 4 to 5 g. of myosin, which means that F-actin with a molecular weight of about 3×10^6 combines with 25–30 myosin molecules (Gergely and Kohler, 1957).

Combination with actin is one of the most sensitive properties of myosin, as is its ATPase activity. Both properties, however, can be abolished without a significant change in solubility. Inactivation of myosin ATPase, and the disappearance of its ability to combine with actin, may occur in a parallel fashion. This has been shown clearly by reacting the –SH groups of myosin in a stepwise fashion (Bailey and Perry, 1947). Treatment of F-actin with the same reagents had little effect on complex formation.

If ATP is added to an actomyosin solution, the complex dissociates into its component proteins. Pyrophosphate, inorganic triphosphate in presence of magnesium and a number of other triphosphorylated nucleotides also have a dissociating action. The dissociation of actomyosin by ATP was demonstrated in a direct fashion in the ultracentrifuge (Portzehl *et al.*, 1950; Johnson and Landolt, 1951), and myosin can be separated from actin by differential ultracentrifugation (A. Weber, 1956). The amount of ATP required for dissociation is small (Mommaerts and Hanson, 1956), and was estimated from viscosity studies to be near to 1 mole per mole of the myosin monomer. The estimation of the association constant of myosin to actin gave a value of the order of

10^{6}, taking into consideration that 25 moles of myosin may be bound by 1 mole of actin (Gergely and Kohler, 1957). The binding may be a temperature dependent equilibrium reaction and, with high gravitational field and at lower temperatures, dissociation of actomyosin was observed (Laki *et al.*, 1952).

At low salt concentration, actomyosin contracts on addition of ATP (A. Szent-Györgyi, 1941). The details of this reaction are discussed in another chapter of this volume (Chapter II).

C. Tropomyosin

1. *General Properties and Isolation*

Tropomyosin is an important fibrous protein of the myofibril which was discovered and characterized by Bailey (1948). It appears to be a universally occurring component of the myofibril and has been prepared from a wide variety of muscles. Tropomyosin comprises about 5% of the myofibrillar proteins of rabbit muscle. No enzymatic activity has been shown to be associated with this protein, and its contribution to the activity of the muscle cell is not known. Its major importance is rather from the more general point of view of protein structure since it is a very well characterized fibrous protein from which reproducible and well defined preparations can easily be obtained (Bailey, 1948). Tropomyosin is remarkably resistant to acid, alkali, or heat treatment. It is not easily denatured at surfaces or by precipitation with nonpolar solvents and can be stored as a lyophilized powder. All these properties help in obtaining uniform preparations.

Little tropomyosin is extracted from muscle with solvents of low ionic strength, though after extraction, tropomyosin is readily soluble under the same conditions. Best yields can be obtained from muscles which are ethanol and ether dried. The dried muscle is extracted with 1 *M* KCl at pH 7 and the tropomyosin is purified by repeated isoelectric precipitations and ammonium sulfate fractionations. Since tropomyosin contains no tryptophan and hardly any proline, purity of the preparation can be conveniently followed by looking for the presence of these residues (Bailey, 1948).

Tropomyosin can be crystallized easily (Bailey, 1948) and is, at present, the only fibrous protein which forms true crystals with sharp edges and faces. The crystal form varies depending on the source of tropomyosin. Rabbit tropomyosin crystallizes in plates. The crystals have a surprisingly high water content, amounting to about 90%, which

explains their extreme fragility and the difficulties encountered in manipulating them. The preparation obtained according to the original description is homogeneous by the criteria of electrophoresis, diffusion, and sedimentation studies (Bailey *et al.*, 1948).

In the absence of ions, the protein polymerizes and a gel is formed. The viscosity of tropomyosin depends on ionic strength; the greatest decrease takes place in the range between water and 0.01 *M* KCl (Tsao *et al.*, 1951). The maximum of isoelectric precipitation is at pH 5.1.

Tropomyosin extracted by salt extraction without dehydration is associated with nucleic acid, and was denoted as "nucleotropomyosin" (Hamoir, 1951a, b). The nucleic acid content may vary depending on the source. Carp nucleotropomyosin is especially rich in nucleic acids, and may contain about 15% nucleic acid by weight. This association is, however, a loose one, and in electrophoretic measurements, the nucleic acid and tropomyosin travel separately (Perry, 1953; Sheng *et al.*, 1956). The complex may have been formed during extraction.

2. *Size and Shape*

The aggregation and molecular weight of tropomyosins obtained from various animals depend on the ionic strength. The most detailed studies were performed on material obtained from rabbit skeletal muscle (Tsao *et al.*, 1951). The weight of the tropomyosin monomer was obtained in urea or at fairly alkaline pH where depolymerization was complete. A number of different methods, including osmotic pressure determinations, sedimentation, diffusion, fluorescent polarization, and light scattering studies, gave values of 54,000 for the molecular weight of the monomer. In neutral salt solutions, the molecular weight depends on ionic strength and a limiting value of about 60,000 is reached at ionic strength higher than 0.6. The intrinsic viscosity of the monomer was found to be 0.52 and the calculated asymmetry gives a length to width ratio of about 25, assuming 25% hydration. The length was calculated on the basis of viscosity and molecular weight and derived from light scattering measurements. Both methods yielded values somewhat below 400 A. (Bailey, 1954). The light scattering studies indicate a regular end-to-end association of the molecules at lower ionic strength, with the possible formation of dimers. The degree of association of the tropomyosins obtained from different species may differ (Tsao *et al.*, 1956; Kominz *et al.*, 1957b). Molecular weights of 60,000 to 150,000 were obtained, under conditions in which rabbit tropomyosin yields

the monomer, using as sources a number of different species (Tsao *et al.*, 1956). This would indicate that the monomers thus obtained vary considerably and do not bear a simple multiple molecular weight relationship to rabbit tropomyosin. Further studies are needed to establish this important point clearly. In spite of the difference in the ease and extent of polymerization of the different tropomyosins, indicating a variable size and shape, there is little difference in the electrophoretic mobilities (Tsao *et al.*, 1956; Kominz *et al.*, 1957).

D. Paramyosin

Paramyosin is a major protein component of certain specialized muscles of molluscs and annelids. These muscles ("catch muscles") are unique in their ability to maintain tensions for prolonged times with very low metabolic activity, and without the mechanical properties characteristic of the tetanic contractions of other muscles. They have a characteristic small-angle X-ray reflection (Bear, 1944) and electron microscopic periodicity (Hall *et al.*, 1945) arising from the presence of paramyosin.

Paramyosin was originally extracted with weak acid (Hodge, 1952) and later with high concentrations of neutral salts (Bailey, 1956; Locker and Schmitt, 1957). Good preparations can be obtained from muscles dried previously with ethanol and ether (Bailey, 1956; Laki, 1957b). Extraction with salts, if not preceded by the drying of the muscle with ethanol or acetone, extracts considerable amounts of actomyosin in addition to paramyosin (Ruegg, 1957). Selective denaturation of actomyosin with ethanol or acetone, or removal of actomyosin by differential centrifugation at high gravitational field, is a simple way to obtain a homogeneous preparation. Preparations free from actomyosin contaminations have been only recently obtained (Bailey, 1956). Paramyosin, if devoid of actomyosin, has no ATPase activity and does not combine with actin. The presence of paramyosin in the "catch muscles" only may suggest a rôle for this unique protein.

Paramyosin is readily soluble at higher ionic strengths at neutral or slightly alkaline pH values. In contrast to tropomyosin, it precipitates at low ionic strengths ($\Gamma/2 \leq 0.3$) in slightly acid solutions (pH $= 6.5$) in the form of needle-shaped crystals. In the absence of ions, it forms a gel. Paramyosin salts out at lower ammonium sulfate concentrations than tropomyosin and precipitation is complete at around 30% saturation. It is not denatured by precipitation with ethanol or acetone, and the material can be stored as a lyophilized powder.

Detailed studies on the size and shape of paramyosin have been reported only on material obtained from *Pinna nobilis* (Kay, 1958). The intrinsic viscosity of 2.0 indicates a very pronounced asymmetry of the molecules. A molecular weight of 137,000 was obtained from light scattering data, and 131,000 from sedimentation and diffusion studies. The preparation appeared to be homogeneous, as judged from sedimentation, diffusion, and electrophoretic studies. Light scattering studies indicated a length of 1400 A. This value agrees with the fundamental repeat period obtained on paramyosin crystals with the aid of the electron microscope (Hodge, 1952). The rigidity and great asymmetry is a remarkable property of the molecule.

E. OTHER "MYOFIBRILLAR" PROTEINS

A number of proteins have been reported to be present in muscle extracts of high ionic strength which, as judged from their electrophoretic mobility and solubility, are different from myosin or actomyosin. The extractability of some of these depends on whether fresh muscle or muscle in rigor is used as starting material. Further work is required to establish their separate identity and for their unambiguous characterization.

1. *Delta Protein*

The most extensive study was performed on the delta protein (Amberson *et al.*, 1957). This protein was identified as a faster moving component than myosin in the electrophoretic experiments. For its extraction, high ionic strength is required. Extractability is facilitated by the presence of inorganic pyrophosphate. Part of the protein coprecipitates with myosin and can be separated from it by treatment with mersalyl. The protein appears to be a fairly asymmetric one which sediments with a sharp peak and has an intrinsic viscosity of about 1.3 (White *et al.*, 1957). It is soluble at low ionic strength and appears to dissociate into smaller units in acid solutions. Films prepared from delta protein give periodicities with spacings at a distance of 165 A in the electron microscope. No enzymatic activity has been detected using purified preparations. The similarities in electrophoretic mobilities and solubility suggest a relationship to tropomyosin, though the higher sedimentation rate and viscosity would indicate an aggregated form. Viscosity, sedimentation, and electrophoretic experiments were interpreted as indicative of some complex formation between myosin and delta protein (Bensusan *et al.*, 1957).

2. *Contractin or Myosin Y*

Electrophoretic analysis of a high salt extract of fatigued muscle or muscle in rigor shows the presence of a component with a lower mobility than myosin (Dubuisson, 1948). Contractin has been separated from actomyosin based on its higher solubility at $\Gamma/2 = 0.3$ and its precipitation at $\Gamma/2 = 0.03$ (Schapira *et al.*, 1957). Contractin has no ATPase activity and does not combine with actin. Its viscosity is below that of myosin.

3. *Metamyosin*

Metamyosin is an electrophoretic component of salt extract of muscle with a lower mobility than myosin or contractin (Raeber *et al.*, 1955). Relatively myosin-free solution may be obtained by using muscle in rigor as starting material. Fetal muscle appears to contain more metamyosin than adult muscle. Metamyosin does not combine with actin and no enzymatic activity has been detected in purified preparations.

4. *X Protein*

X protein designates proteins other than myosin which are extracted with solvents which remove myosin from a well-washed glycerinated myofibril (A. G. Szent-Györgyi *et al.*, 1955). Further studies indicated an association of part of the components present in the extract at low ionic strength (Villafranca, 1956). The extract in the preparation contains more than one component, and the presence of tropomyosin in the preparation has been reported (Corsi, 1957).

III. ANALYSIS OF FIBROUS MUSCLE PROTEINS

A. Amino Acid Composition

Careful studies were conducted using ion exchange chromatography (Moore and Stein, 1951) to obtain the amino acid composition of rabbit myosin, actin, tropomyosin, and meromyosins (Kominz *et al.*, 1954) (Table III). There is an extensive comparative study on the amino acid content of tropomyosins and paramyosins obtained from a great variety of species (Yoshimura, 1955; Bailey, 1957; Jen and Tsao, 1957; Kominz *et al.*, 1957b;) (Tables IV, V). Titration data are available for myosin (Mihályi, 1950) and the meromyosins (Nanninga, 1955a, b) (Table VI).

TABLE III

AMINO ACID COMPOSITION OF FIBROUS PROTEINS OF RABBIT MYOFIBRIL[a]
(Results expressed as moles per 10^5 g. protein)

Amino acid	Actin	Tropomyosin	Myosin	LMM	HMM
Cystine/2	11.2	6.5	8.6	5.6	10.9
Aspartic acid	82	89	85	77	88
Threonine	59	28	41	38	49
Serine	56	40	41	37	43
Glutamic acid	101	211	155	174	138
Glycine	67	12.5	39	21	45
Proline	44	0	22	8.4	29
Alanine	71	110	78	76	73
Valine	42	38	42	39	45
Methionine	30	16	22	14	19
Isoleucine	57	29	42	35	42
Leucine	63	95	79	85	78
Tyrosine	32	15	18	12	21
Phenylalanine	29	3.5	27	9.6	40
Histidine	19	5.5	15	19	11.5
Lysine	52	110	85	83	82
Arginine	38	42	41	51	29
Tryptophan	10	0	4	6	3
Amide NH_3	(66)	(64)	(86)	(107)	(100)
Totals	863	851	844	794	847
Free acidic groups	117	236	156	?144	?126
Basic groups	109	157.5	141	153	123
Polar groups	450	547	492	497	473

[a] Data from Kominz *et al.* (1954).

TABLE IV

AMINO ACID COMPOSITION OF TROPOMYOSINS
(Results expressed as moles per 10^5 g. protein)

Amino acid	Busycon[1]	Venus[1]	Loligo[1]	Squid[2]	Homarus[1]	Bovine, human, rabbit[3] uterine; bovine urinary bladder	Rabbit[3] skeletal bovine heart
Cystine/2	—	—	—	6	—	—	6.5 (rbt)
Aspartic acid	107	110	103	100	101	82	88–89
Threonine	31	35	38	33	27	26	25–26
Serine	48	43	45	55	43	40	40–41
Glutamic acid	204	205	195	212	200	213–215	212–213
Proline	1.8	1.7	4	0	3	0.9–2.1	1.7
Glycine	20	20	17	28	14	8–11	9–12.5
Alanine	101	101	93	95	90	107–108	107–110
Valine	21	40	24	25	33	27	27
Methionine	18	8	12	17	22	22–23	16
Isoleucine	24	27	25	20	12	24–27	30
Leucine	100	88	86	95	88	94–95	95
Tyrosine	15	20	10	8	12	11–13	15
Phenylalanine	8	6.3	12	8	11	3.5–4.8	3.3–3.7
Histidine	?3	3.3	?3	12	?3	8–11	5.3–5.5
Lysine	79	78	78	85	78	93	104–107
Arginine	59	67	63	69	63	48–50	41–42
Tryptophan	0	—	—	—	0	—	0
Amide NH_3	(90)	(84)	(85)	(95)	(90)	(60–63)	(62–64)
Totals	840	852	808	862	800	808–828	826–842
Free acidic groups	221	231	213	217	211	235	238
Basic groups	141	148	144	166	144	151	152
Polar groups	546	561	535	580	527	525	540

[1] Kominz *et al.* (1957b).
[2] Yoshimura (1955).
[3] Kominz *et al.* (1957a).

TABLE V

AMINO ACID COMPOSITION OF PARAMYOSINS
(Results expressed as moles per 10^5 g. protein)

Amino acid	Lumbricus[1]	Arenicola[1]	Busycon[1]	Venus[1]	Pinna[2]	Octopus[1]
Cystine/2	—	—	—	—	2.5	—
Aspartic acid	97	120	112	114	111	91
Threonine	40	33	40	36	22	37
Serine	51	44	51	39	49	52
Glutamic acid	169	156	164	169	173	160
Proline	1.7	4	3.7	1.5	0	0
Glycine	25	25	19	15	12	16
Alanine	86	87	91	108	102	79
Valine	29	30	29	28	36	32
Methionine	1.5	13	11	11	15.5	13
Isoleucine	35	25	20	22	28	34
Leucine	124	106	99	106	103	104
Tyrosine	10	10	12	18	14	8
Phenylalanine	12	6	8	6	8.5	6.5
Histidine	10	6	? 6	? 4	4.5	11
Lysine	56	56	55	59	65	66
Arginine	86	85	81	81	79	70
Tryptophan	0	0	0	0	0	0
Amide NH_3	(98)	(113)	(112)	(110)	(116)	(92)
Totals	833	806	802	817	825	780
Free acidic groups	168	163	164	173	168	159
Basic groups	152	147	142	144	148	147
Polar groups	519	510	521	520	519	495

[1] Kominz *et al.* (1957b).
[2] Bailey (1957).

TABLE IV

AMINO ACID COMPOSITION OF TROPOMYOSINS
(Results expressed as moles per 10^5 g. protein)

Amino acid	Busycon[1]	Venus[1]	Loligo[1]	Squid[2]	Homarus[1]	Bovine, human, rabbit[3] uterine; bovine urinary bladder	Rabbit[3] skeletal bovine heart
Cystine/2	—	—	—	6	—	—	6.5 (rbt)
Aspartic acid	107	110	103	100	101	82	88–89
Threonine	31	35	38	33	27	26	25–26
Serine	48	43	45	55	43	40	40–41
Glutamic acid	204	205	195	212	200	213–215	212–213
Proline	1.8	1.7	4	0	3	0.9–2.1	1.7
Glycine	20	20	17	28	14	8–11	9–12.5
Alanine	101	101	93	95	90	107–108	107–110
Valine	21	40	24	25	33	27	27
Methionine	18	8	12	17	22	22–23	16
Isoleucine	24	27	25	20	12	24–27	30
Leucine	100	88	86	95	88	94–95	95
Tyrosine	15	20	10	8	12	11–13	15
Phenylalanine	8	6.3	12	8	11	3.5–4.8	3.3–3.7
Histidine	?3	3.3	?3	12	?3	8–11	5.3–5.5
Lysine	79	78	78	85	78	93	104–107
Arginine	59	67	63	69	63	48–50	41–42
Tryptophan	0	—	—	—	0	—	0
Amide NH_3	(90)	(84)	(85)	(95)	(90)	(60–63)	(62–64)
Totals	840	852	808	862	800	808–828	826–842
Free acidic groups	221	231	213	217	211	235	238
Basic groups	141	148	144	166	144	151	152
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[1] Kominz *et al.* (1957b).
[2] Yoshimura (1955).
[3] Kominz *et al.* (1957a).

groups reacting with the reagent. The very high amide ammonia values obtained for LMM and HMM makes such a comparison difficult.

The number of cross linkages in muscle proteins appears to be low. The determination of the free SH groups gives a value which does not differ much from the cysteic acid content obtained after performic acid oxidation. The difference between the two values may give an estimation of the maximum amount of sulfur cross linkages. One tropomyosin monomer has one cross linkage, while the rest of the fibrous muscle proteins have less than one cystine per 10^5 g. Performic acid oxidation does not split tropomyosin into two chains (Locker, 1954). The low number of cross linkages may be one reason for the relatively easy solubilization of the fibrous muscle proteins, as compared to keratin.

The extensive comparative studies of the amino acid composition of tropomyosin and paramyosin established certain similarities in the amino acid pattern of these two proteins (Bailey, 1957; Kominz *et al.*, 1957b); this led to the suggestion that the two proteins are identical. The outstanding common features are the absence of tryptophan and the very low proline content. The major difference between tropomyosin and paramyosin resides in the distribution and concentration of the dibasic and dicarboxylic acids. The glutamic acid content of the various paramyosins is lower than that of the various tropomyosins by about 40–50 groups per 10^5 g. The amide ammonia approximates the aspartic acid content in paramyosin, but it is about 15 to 20 groups less in tropomyosin, with the possible exception of squid. The lysine content of the various paramyosins is about 60 residues. Tropomyosin obtained from invertebrates has a lysine content of about 80 residues. Rabbit tropomyosin is characterized by a lysine content of 107 residues. The number of arginine residues varies from 70 to 86 in the paramyosins, from 59 to 63 in tropomyosins obtained from invertebrates, and is 41 in rabbit tropomyosin. If paramyosin and tropomyosin from the same species are compared, there are differences in four amino acids other than the residues mentioned here.

There is a variation in the number of residues of several amino acids if the paramyosins obtained from various sources are compared, but it is difficult to see a consistent trend. The same is true for tropomyosins.

It is of interest that while bovine heart and rabbit skeletal muscle tropomyosins are apparently identical in their amino acid composition, there is some difference in the tropomyosins obtained from smooth

TABLE VI

TITRATION VALUES OF MUSCLE PROTEINS

Protein		Titration	Analytical[1]
Myosin[2]:	Carboxyl groups	165	156
	Bases	150	141
	Histidine	16	15
	Lysine	91	85
	Arginine	43	41
LMM[3]:	Carboxyl groups	182	144
	Bases	168	153
	Histidine	20	19
	Lysine	64	83
	Arginine	83	51
	Tyrosine	14	12
HMM[3]:	Carboxyl groups	163	126
	Bases	131	123
	Histidine	8	11.5
	Lysine	80	82
	Arginine	43	29
	Tyrosine	22	21

[1] Kominz *et al.* (1954).
[2] Mihályi (1950).
[3] Nanninga (1955a, b).

There are certain common features in the amino acid pattern characterizing all the fibrous muscle proteins. The most remarkable feature is the high concentration of charged side chains. The over-all pattern of all the four proteins is not too dissimilar. Tropomyosin has the highest charge density, with two out of three residues bearing a positive or negative charged group. The anionic groups are in a slight excess to the cationic ones in all of the fibrous muscle proteins, the isoelectric points are between pH 5 and pH 6, and the net charge at pH 7 is not too large.

The concentration of charged groups measured titrimetrically on myosin exceeds the values calculated from the analytical measurements. Thus titration gives about 10 to 15 more basic and acidic groups in 10^5 g. myosin The discrepancy may be accounted for by the presence of α-amino groups not considered in the values obtained from amino acid analysis. The fact that C- and N-terminal end groups are not present in such high concentrations opposes such an idea; still, we do not know as yet whether some type of masking does not prevent the

TABLE VII

C—TERMINAL ENDS AND SEQUENCES OF FIBROUS MUSCLE PROTEINS

Protein	Carboxypeptidase method	Hydrazine method
Actin[1]	phe-isoleu-his	phe
Myosin[1]	isoleu	?
Tropomyosin[1] (rabbit skeletal)	isoleu-ser-thre-meth-isoleu-ala	isoleu
Tropomyosin[2] (rabbit, skeletal, human, bovine bladder)	isoleu ser	
Tropomyosin[2] (human uterus)	asp leu	

[1] Locker (1954).
[2] Kominz *et al.* (1957a).

C-terminal end of the molecule with carboxypeptidase. Hydrazinolysis, too, showed the presence of isoleucine only. This would indicate that tropomyosin, as far as the C-terminal end is concerned, is a single chain, and from the relative rate of the appearance of the residues, a sequence of isoleu-ser-thre-met-isoleu-ala was proposed (Locker, 1956). A different result, using the carboxypeptidase technique, indicated the presence of two open chains at the C-terminal end of tropomyosin (Kominz *et al.*, 1957c). It was concluded that the C-terminal ends are isoleucine and serine in rabbit, human skeletal, and bovine bladder *tropomyosins*, and asparagine and leucine in human uterus tropomyosin.

In paramyosin, 1 mole of total N-terminal groups in 500,000 g. protein was measured (Bailey, 1957).

The sequence at the C-terminal end of actin was proposed to be phe-isoleu-his, based on studies with carboxypeptidase. *Hydrazinolysis* showed only the presence of phenylalanine. The concentration of C-terminal end groups was about one mole per 70,000 g. (Locker, 1954).

The situation with myosin is less clear. One isoleucine could be observed per 300,000 g. with the carboxypeptidase method, though other residues also accumulated in significant quantities. *With hydrazinolysis*, a number of different residues in about equal quantities were shown to be present (Locker, 1954). The water-soluble fragment of

muscle. Thus the tropomyosins of rabbit uterus and bovine bladder muscle have a lower lysine and higher arginine content than the tropomyosins obtained from skeletal muscles of these animals (Kominz *et al.*, 1957c).

As a consequence of the consistent differences in the amino acid composition of paramyosins and tropomyosins, all paramyosins have a considerably lower electrophoretic mobility at neutral pH values than tropomyosins, though the mobilities, within the groups, are fairly constant (Tsao *et al.*, 1956; Kominz *et al.*, 1957b).

It is likely that tropomyosin and paramyosin belong to the same class of proteins. Tropomyosin is universally present in all types of muscle studied. The occurrence of paramyosin is restricted to certain types of invertebrate muscle characterized by a unique small-angle X-ray diffraction pattern and electron microscopic periodicity. In these muscles, tropomyosin and paramyosin are both present simultaneously, and they show characteristic differences. Thus it is more convenient and perhaps less confusing to retain the original nomenclature.

B. End Group and Sequence Studies

N-terminal group studies were conducted using the Sanger's (1945) fluorodinitrobenzene technique on myosin, tropomyosin, paramyosin, and meromyosins. C-terminal groups and short sequences were obtained for actin, myosin, tropomyosin, meromyosins, the components obtained after urea and bicarbonate treatment using carboxypeptidase (Lens, 1949), and hydrazinolysis (Akabori *et al.*, 1952) (Table VII).

There is a curious difference in the results obtained on the intact proteins when the N-terminal and C-terminal groups are compared. All the fibrous muscle proteins are in general characterized by the absence of N-terminal groups in a mole to mole ratio (Bailey, 1951), while the presence of C-terminal groups can be readily shown to be in such ratios (Locker, 1954).

In tropomyosin, the concentration of N-terminal groups decreases with purification and appears to reach a limiting value of 1 mole of total N-terminal groups in each 400,000 g. This is not a single residue, but is composed of a number of different amino acids. With myosin the situation is somewhat similar, there is 1 N-terminal group for each 800,000 g. and the end groups are made up of a number of different residues (Bailey, 1951).

One mole of isoleucine per mole of tropomyosin was found at the

al., 1958). It should be kept in mind that myosin contains a large number of basic or aromatic residues, the hydrolysis of which may not necessarily be connected with the liberation of meromyosins.

If LMM is depolymerized by urea, there is a considerable increase in both the N-terminal and C-terminal groups. In the case of urea-depolymerized LMM Fr. 1, the chainweight and molecular weight agree and gives a value of about 5,000. Again, the pattern of the amino acid residues contributing to the terminal ends is similar to that of the intact LMM, and the basic or aromatic residues comprise only a small fraction of the C-terminal residues (Middlebrook, 1958).

In conclusion, the presence of masked end groups in myosin is a possibility which should be considered. We do not know definitely, as yet, what is the mechanism of the fragmentation of the myosin molecule by proteolytic enzymes, or what is the exact nature of the linkage holding the meromyosins together. The unusual features of the process suggest an unusual situation.

IV. POLYPEPTIDE CHAIN CONFIGURATION OF THE FIBROUS MUSCLE PROTEINS

A. Wide-Angle X-Ray Studies

The fibrous proteins of muscle exhibit two types of wide-angle X-ray pattern. Myosin, tropomyosin, and paramyosin are members of the KMEF class of proteins, while actin has a unique pattern and is not a member of the KMEF class.

The 5.1 A meridional spacing, characterizing the α-proteins, has been observed on oriented films from myosin (Astbury and Dickinson, 1940), tropomyosin (Astbury *et al.*, 1948), and paramyosin (Beighton, 1956). The same spacing was obtained from fibers prepared from gels of the same proteins and of the meromyosins (Cohen and A. G. Szent-Györgyi, 1957). The α-pattern is the only regularly occurring configurational feature of the polypeptide chains in the native state of these proteins.

The α-configuration is a folded one and can be converted by stretching the oriented specimen to about twice its original length into an extended β-configuration, which is characterized by an intense equatorial reflection at 4.6 A. Myosin provides one of the classic examples of such a configurational change (Astbury and Dickinson, 1940). With suitable heat treatment, the preparation may exhibit the cross β-pattern

myosin, obtained after bicarbonate treatment, contained 1 C-terminal isoleucine for a molecular weight of 29,000 (Kominz *et al.*, 1958). The water-soluble fraction obtained after urea treatment contains several different free amino acid residues, whose sum is equivalent to 1 N-terminal residue per 16,000 (Tsao, 1953a).

The absence of N-terminal residues in a mole to mole ratio may mean that those which are present originate from tenacious impurities present in small concentrations. The possibility also exists, though, that the N-terminal groups are present, but masked. There are observations which suggest such a possibility. The concentration of N-terminal groups doubles when myosin is treated with urea (Tsao, 1953a; Middlebrook, 1958). The concentration of the N- and C-terminal groups on the meromyosin is considerably increased as compared with myosin. Thus LMM obtained after trypsin treatment has a chainweight of about 60,000, the terminal groups being composed of a number of different amino acid residues, none of them at a concentration high enough to reach a mole to mole ratio (Middlebrook, 1958). The chainweight of HMM is similarly increased. The simplest explanation to interpret this increase in N-terminal group concentration is to assume a peptide bond hydrolysis. If the action of the proteolytic enzymes is a direct hydrolysis of a peptide linkage connecting the meromyosins, one would expect the presence of a basic C-terminal group after trypsin treatment, and the presence of an aromatic C-terminal group after chymotrypsin treatment, on one of the meromyosins in a mole to mole ratio. Furthermore, the presence of a single type of N-terminal group on one of the meromyosins would also be likely. Such an interpretation is not confirmed by the results of C-terminal group analysis of the meromyosins with either the carboxypeptidase method or Akabori's hydrazinolysis. The chainweight as determined from the C-terminal group analysis increases after the enzyme treatment, but the C-terminal groups are made up of about eight different amino acid residues. Apart from the difference in the chainweight, the amino acid pattern contributing to the C-terminal or N-terminal ends is undistinguishable using trypsin, chymotrypsin, or subtilisin to produce the meromyosins. Lysine or arginine, expected from tryptic hydrolysis, or phenylalanine or tyrosine, expected after chymotryptic hydrolysis, are present in concentrations less than 0.1 mole per mole of protein (Middlebrook, 1958). In another study, differences in the C-terminal ends of the meromyosin obtained after chymotryptic treatment and after tryptic treatment, have been reported (Gergely *et*

constants. Negative sign of b_0 indicates that the helix has a right-handed sense of twist; its value depends on the extent of α-helical configuration. With denatured proteins or with proteins not having the α-helical structure, its value is zero. The equation has been successfully applied to synthetic polypeptides (Moffitt and Yang, 1956; Doty and Lundberg, 1957) and to proteins (Doty and Yang, 1957; Cohen and A. G. Szent-Györgyi, 1957). The dispersion measurements on α-proteins are particularly useful since the presence of α-helical configuration can be independently shown by the wide-angle X-ray diffraction studies. In general, the fibrous muscle proteins have a higher helix content than the globular proteins, as calculated from the studies of optical rotation (Cohen and A. G. Szent-Györgyi, 1957) (Table VIII). The dispersion curves of all the fibrous α-proteins of muscle are anomalous. Such anomality can be predicted from Moffitt's equation if the helix content

TABLE VIII

ROTATORY DISPERSION OF FIBROUS MUSCLE PROTEINS[1]

Protein	Native		Denatured in 9.5 M urea		Helix (wt. %)	Cystine[2] (in 10^5 g.)	Proline (wt. %)
	$[\alpha]_{5780}$	b_0	$[\alpha]_{5780}$	λ_0 (A.)			
LMM Fr. 1	−13.0°	−660°	−118°	2120	100	0	0.22
Tropomyosin	−16.0°	−620°	−118°	2130	94	1.65	0.35
Paramyosin	−11.1°	−600°	− 63°	—	91	0	0.21
LMM	−20.4°	−490°	−107°	2150	74	0.65	0.97
Myosin	−28.7°	−370°	−108°	2180	56	0.6	2.08
HMM	−34.5°	−300°	−103°	2150	45	1.2	2.87

[1] Cohen and A. G. Szent-Györgyi (1957).
A. G. Szent-Györgyi and Cohen (1957).
[2] A. G. Szent-Györgyi *et al* (1959).

of the macromolecule exceeds 50%. There is a difference in the dispersion curves within the individual α-proteins in the native state which disappears in high urea concentrations. From the value of constant b_0, the helix contents were estimated. LMM Fr. 1, tropomyosin from rabbit and paramyosin from *Venus mercenaria* gave values similar to synthetic polypeptides in solvents favoring helical configuration. The helix content of these three proteins appeared to be higher than 90%. The lowest value was obtained for HMM, which may be of interest since this is the component of myosin which carries the ATPase activity

in which the 4.6 A. reflection shows on the meridian, and which is interpreted as indicating that the chains are oriented at right angles to the direction of the stretch.

The presence of the 5.1 A. reflection is significant and indicates that the fibrous muscle proteins contain regions in the α-helical configuration. The 5.1 A. reflection indicates also that these regions are not simple α-helices but can be described rather as cables of α-helices in a supercoiled configuration (Crick, 1953; Pauling and Corey, 1953) consisting of more than one parallel polypeptide chain, as opposed to synthetic polypeptides in the α-helical configuration which consist of single chains and show a meridional reflection at 5.3 A.

Oriented films of actin (Astbury *et al.*, 1947) or oriented fibers prepared from F actin (Cohen and Hanson, 1956) *do not show* the 5.1 A. meridional reflection; the diffraction pattern exhibits a diffuse ring at 4.6 A. This protein, therefore, cannot be fitted into any existing class of proteins on the basis of wide-angle studies. There is no evidence, as yet, as to what is the polypeptide chain configuration which would be compatible with the observed spacings.

B. Optical Rotation

Studies of rotary dispersion may show the presence of helical polypeptide chain configuration, determine the sense of twist of the helix, and estimate the extent of helix content of proteins. The information obtained from rotatory activity is an important corollary to the wide-angle X-ray diffraction studies, which cannot be used directly to estimate the extent of crystalline regions of the molecules. It had been pointed out some time ago that the differences in the specific rotation of denatured and native proteins may arise from the helical configuration in the native state (Cohen, 1955) (see Chapter VII, Volume I). It is only recently, though, that the theory of the rotary dispersion of helical macromolecules has been worked out and a quantative estimation of helix content has become possible (Moffitt, 1956; Moffit *et al.*, 1957). The following equation describes the rotary dispersion of a fully coiled α-helix:

$$[\alpha]_\lambda = \left(\frac{100}{M}\right)\left(\frac{n^2+2}{3}\right)\left[\frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2}+\frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right]$$

where M is the average residue weight, n is the refractive index of the medium, λ is the wave length, λ_0 equals 2100A., a_0 and b_0 are

constants. Negative sign of b_0 indicates that the helix has a right-handed sense of twist; its value depends on the extent of α-helical configuration. With denatured proteins or with proteins not having the α-helical structure, its value is zero. The equation has been successfully applied to synthetic polypeptides (Moffitt and Yang, 1956; Doty and Lundberg, 1957) and to proteins (Doty and Yang, 1957; Cohen and A. G. Szent-Györgyi, 1957). The dispersion measurements on α-proteins are particularly useful since the presence of α-helical configuration can be independently shown by the wide-angle X-ray diffraction studies. In general, the fibrous muscle proteins have a higher helix content than the globular proteins, as calculated from the studies of optical rotation (Cohen and A. G. Szent-Györgyi, 1957) (Table VIII). The dispersion curves of all the fibrous α-proteins of muscle are anomalous. Such anomality can be predicted from Moffitt's equation if the helix content

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of the macromolecule exceeds 50%. There is a difference in the dispersion curves within the individual α-proteins in the native state which disappears in high urea concentrations. From the value of constant b_0, the helix contents were estimated. LMM Fr. 1, tropomyosin from rabbit and paramyosin from *Venus mercenaria* gave values similar to synthetic polypeptides in solvents favoring helical configuration. The helix content of these three proteins appeared to be higher than 90%. The lowest value was obtained for HMM, which may be of interest since this is the component of myosin which carries the ATPase activity

and combines with actin. It may well be that these centers do not participate in the helix. From the b_0 constant obtained for actin, one may estimate a helix content amounting to about 30%, though in this case there is no independent evidence showing the presence of the α-helical configuration (Cohen, 1956). The helix content varies inversely with proline content, indicating that proline has a major role in determining the extent of helical configuration (A. G. Szent-Györgyi and Cohen, 1957).

LMM Fr. 1, tropomyosin, and paramyosin behave as fully coiled helices. Their high helix content supports the validity of calculations for the length of the molecules based on a rise of 1.5 A. per residue. A comparison of these calculations with the length obtained by other techniques may give clues to the number of parallel peptide chains present.

Tropomyosin is particularly suited for such calculations because of the reliable data at hand (Bailey, 1954). In a tropomyosin monomer with a molecular weight of 53,000, there are 455 residues. The maximum length calculated on the basis of the 1.5 A. rise per residue in an α-helix is 682 A. From light scattering data, the length of the molecule is 385 A. The intrinsic viscosity indicates an asymmetry of 25, assuming 25% hydration. From this value, a length of 365 A. can be calculated. It is obvious that a two chain model gives the best fit for the tropomyosin molecule (Bailey, 1954).

The maximum length of LMM Fr. 1 would be 1570 A., based on a molecular weight of around 120,000 (Cohen and A. G. Szent-Györgyi, 1958). The asymmetry derived from an intrinsic viscosity of 1.0 is 37, assuming 25% hydration. The calculated length for a prolate ellipsoid is about 700 A. It appears that a two chain model fits this molecule also, though independent measurements of the length are desirable.

The molecular weight of paramyosin from *Pinna nobilis* was found to be 137,000 (Kay, 1958). Light scattering data indicated a length of 1400 A. These measurements agree with the basic repeat period of 1400 obtained in the electron miscroscope (Hodge, 1952). The maximum length calculated on the basis of the α-structure and molecular weight is 1700 A. That would lead to the surprising conclusion that a considerable portion of the molecule is a single chain. How it can exist in a supercoiled configuration which is supposed to be a result of the interaction of more than one chain is not yet clear. Further studies on paramyosin will be of great interest.

C. Small-Angle X-Ray and Electron Microscopic Studies

Actin, LMM, LMM Fr. 1, tropomyosin, and paramyosin exhibit a specific pattern in small-angle X-ray diffraction and electron optical studies, which allows the characterization of these proteins on the basis of their periodic structures.

All types of muscles show reflections corresponding to a fundamental fiber period of about 400 A. based on small-angle X-ray studies on dried preparations (Bear, 1945). With living muscles, additional lower orders were also obtained (Huxley, 1953). Small-angle X-ray studies on oriented F-actin films show a great similarity in the position of the spacings with that of the characteristic pattern of muscle (Astbury *et al.*, 1947). The pattern obtained from spun fibers prepared from F-actin (Cohen and Hanson, 1956) agrees well with the muscle pattern in the position of the spacings. The differences observed relate to the relative intensities of the layer lines. Thus, the small-angle X-ray pattern of muscle, with the possible exception of the 400 A. spacing, appears to originate from actin.

In a detailed study of the reflections as present in molluscan muscles, actin was described as a two dimensional net with cell dimensions of 82 and 406 A. and an 82° angle between the axes, or as a large scale helix which is formed by the actin rods containing 15 nodes on 7 turns and forming an axial period of 406 A. (Selby and Bear, 1956). The width of the actin filaments was deduced to be 50 to 100 A. Here again, reflections at 400 A. and 195 A. did not clearly belong to the actin net system and other macromolecular components of a similar axial periodicity could contribute to their presence. The electron micrographs of F-actin show a filament width of about 100 A. with the filaments having an oblique striation with a periodicity of 300 A. (Rozsa *et al.*, 1949). The correlation of these striations with the small-angle X-ray pattern is not clear.

The small-angle X-ray pattern of the white adductor of *Venus mercenaria* was studied in detail (Bear and Selby, 1956). 25 reflections were observed and interpreted as a result of a two dimensional net. The dimensions of the net cell are: a = 250 A., b = 720 A.; the angle between the axes was 90.5°. There are five nodes for a 720 A. fiber period and the subperiodicity is 144 A. The paramyosin rods making up the net are super helices and have a diameter of 100 to 125 A. An alternative interpretation of the paramyosin diffraction positions and maxima

described the paramyosin component of these muscle fibrils in terms of helices with five equivalent scattering units which are arranged in two helical turns along a 725 A. repeat period on the fibril axis (Bear and Selby, 1956). In the electron optical studies, a periodicity of 145 A. was observed (Hall *et al.*, 1945). The lines were made up of spots with a separation of 193 A. transverse to the fiber axis. The arrangement of the spots was such that it gave rise to a true repeat period of 720 A. The identity period observed in the small-angle X-ray studies is in close agreement with the electron optical studies.

Extracted and reconstituted paramyosin gives a different repeat period, corresponding to 1400 A. (Hodge, 1952). Between these periods, about 15 differently staining segments can be seen. The periodicity depends on the experimental conditions; recently, it was possible to obtain the 145 A. periodicity of the paramyosin-containing muscles with extracted and reconstituted paramyosin preparations (Hodge, 1958). All the periodicities bear a direct relationship to the 1400 A. repeat period and it is probable that the differences are due to the parallel, antiparallel, or staggered arrangement of the paramyosin molecules, the length of which is 1400 A. No good small-angle X-ray diffraction pattern has been reported as yet for films or fibers prepared from paramyosin solutions. A 70 A. spacing was observed on films prepared from paramyosin crystals (Beighton, 1956).

Tropomyosin crystals show a repeat period at somewhat below 200 A. A subperiod is present lying centrally between the main periods. In some instance, PTA stained material may form a net of squares, each side of the square amounting to either 200 A. or 400 A., while the inner part seems to be empty (Hodge *et al.*, 1958). This may be connected with the large hydration of tropomyosin crystals. The periodicity in the electron miscroscope again is in a simple relationship with the length of the molecule. A small-angle X-ray diffraction pattern shows reflections at 25 and 45 A. distances, but is not detailed enough to ascertain the axial periodicity.

Myosin does not show any periodicity in electron optical studies; neither has there been any small-angle reflection reported. This lack of periodicity is in contrast to the behavior of its LMM component. In electron optical studies, unstained and unshadowed LMM shows very intense lines at 420 A. distances. Subperiods consisting of faint lines are present between the main periods (Philpott and A. G. Szent-Györgyi, 1954). LMM Fr. 1 has the same periodicity and PTA staining brings

out the subperiodicity showing five lines with about 70 A. separation. The intense lines are probably the result of accumulation of salt. Crystals stained with PTA in the absence of KCl show only the subperiods and the main periodicity is absent. In small-angle X-ray studies, LMM or LMM Fr. 1 fibers show about 10 reflections with a true axial periodicity of 428 A., in good agreement with the electron optical studies (Cohen and A. G. Szent-Györgyi, 1958). The lack of crystalline regions in myosin may be the result of the presence of HMM, which prevents the regular alignment of the molecules.

There are certain interesting aspects of the electron optical and small-angle X-ray studies. All the highly helical molecules, paramyosin, tropomyosin, paramyosin, and LMM Fr 1 can be fingerprinted on the basis of the periodicities that they show. The periodicities are obviously the result of side chain interactions which are permitted by the regularity of the main chain configuration. The recurrent feature is the 400 A. spacing of muscle proteins. The real importance of this dimensional correspondence is not known. It is found in electron optical studies on muscle (Draper and Hodge, 1949), and it is an important reflection and not yet clearly explained in the small-angle X-ray diffraction studies. Actin, LMM, and tropomyosin all seem to be capable of contributing to such a periodicity.

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CHAPTER II

Biochemistry of Muscular Action

D. M. NEEDHAM

I. ENERGY PROVISION IN MUSCLE

A. The Lactic Acid Period

The energy used by muscle in performance of work and maintenance of tension is ultimately derived from chemical reactions going on within it. The nature of these reactions was the subject of much experimentation and controversy during the latter half of the nineteenth century, but quantitative and reproducible results were not obtained until the classical work of Fletcher and Hopkins (1907). They showed clearly for

the first time that fatigue and death rigor are accompanied by lactic acid production. This great step forward was due to their recognition of the need to reduce to a minimum any stimulation of the muscle during fixation and extraction. Later, Parnas and Wagner (1914) showed that the lactic acid is derived from the muscle glycogen. Now it was known that the formation of lactic acid from glycogen is a reaction going on with output of heat—the difference in the heats of combustion of the two substances is 16,300 cal. per gram molecule lactic acid formed, according to Meier and Meyerhof (1924). It seemed reasonable to suppose—and the assumption has proved correct—that here was a reaction providing energy necessary for contraction. The long series of studies in Meyerhof's laboratory afforded support for this view, showing as they did the proportionality during anaerobic contraction between lactic acid formation and tension production or work done (Meyerhof, 1920, 1921).

The experiments on heat production in frog muscle during anaerobic contraction and relaxation (the initial heat) showed that the heat production was much greater than the expected amount—about 35,100 cal. per gram of lactic acid formed. A part of this excess could be explained by neutralization in the tissue, but a discrepancy of some 45% remained until it was explained by the metabolism of phosphagen. The significant fact was discovered that the size of the initial heat (Weizacker, 1914), its distribution in time (Hill and Hartree, 1920), and its relation to tension production (A. V. Hill, 1928b) are the same when the muscle contracts in oxygen as when it contracts in nitrogen. Thus the chemical reactions underlying contraction must be nonoxidative; later D. K. Hill (1940a) showed for frog muscle at 0°C. with a short tetanus that even when conditions are from the beginning aerobic, increased oxygen consumption does indeed only start after activity (contraction and relaxation) is over. The aerobic recovery-heat production is large, almost equal to the initial heat (A. V. Hill, 1928b); but if the conditions are maintained anaerobic, the period after activity shows only small and variable heat production—averaging about 20% of the initial heat, spread over about 30 min. (see D. K. Hill, 1940b).

B. Contraction and Creatine Phosphate

For twenty years the idea of lactic acid formation from carbohydrate as the only energy providing reaction remained unchallenged. Then Lundsgaard (1930a) found that lactic acid formation in muscle could

be stopped by poisoning with iodoacetate, but that nevertheless contraction could go on. He showed that in these poisoned muscles, tension production was proportional to breakdown of creatine phosphate. This substance (phosphagen) had been discovered in muscle by Eggleton and Eggleton (1927a, b) and independently by Fiske and Subbarow (1927, 1929a) who first elucidated its structure. Eggleton and Eggleton (1929–1930) connected creatine phosphate metabolism with contraction, for they found it to decrease in amount during contraction, while resynthesis occurred on recovery in oxygen. Nachmansohn (1928, 1929) showed that even in nitrogen there was rapid resynthesis of about 30% of the creatine phosphate in the 30 sec. immediately after relaxation. At the same time, Meyerhof and Lohmann (1928a, b) found creatine phosphate hydrolysis to be an exothermic reaction, about 12,000 cal. per gram molecule of inorganic phosphate being liberated in the conditions of their experiments. Nachmansohn (1928, 1929), comparing tension development with creatine phosphate disappearance, found that during a succession of contractions, this disappearance is much greater during the early contractions than during the later ones.

These facts concerning creatine phosphate necessitated a review of the lactic acid theory of contraction. In the first place, all experiments up to that time had seemed to show proportionality between tension production, heat liberation and lactic acid formation (see A. V. Hill, 1928a); but if creatine phosphate hydrolysis, an exothermic reaction, was going on to a greater degree at the beginning of a contraction series, a greater heat production at this time was to be expected. Again, some explanation was needed for the rapid anaerobic resynthesis of creatine phosphate immediately after contraction. Here was an endothermic reaction going on during a short space of time when heat exchange was negligible and certainly no equivalent heat absorption could be detected (Hartree and Hill, 1928). The suggestion was made at the time that creatine phosphate was merely "unstabilized" *in vivo*, some change rendering the compound unusually liable to be broken down by the chemical treatment used in estimation (see Hill, 1928a; Hartree and Hill, 1928; Nachmansohn, 1928; Meyerhof *et al.*, 1930).

Then Lipmann and Meyerhof (1930) made the significant observation that, during the first few of a series of short tetani, there is a change in the muscle pH not to the acid but towards the alkaline side. These pH changes were studied in intact uninjured muscle (thin frog sartorii) lying in a bath of bicarbonate Ringer solution with a nitrogen-

CO_2 atmosphere above. They were related by Lipmann and Meyerhof to an earlier study (Meyerhof and Lohmann, 1928b) of the titration curves of creatine phosphate and of an equimolecular mixture of free creatine and inorganic phosphate. Comparison of these curves showed that between pH 3 and 7.5, hydrolysis of creatine phosphate is accompanied by liberation of base; it was striking that the degree of pH change in the muscle on contraction varied according to the pH of the bathing medium and closely paralleled the amount of base liberation to be expected at different pH values from the titration curves. Thus it was shown that creatine phosphate breakdown in the muscle is a reality, and the way was prepared for the results of Lundsgaard.

Lundsgaard (1930a, b) took the point of view that, in muscle poisoned with iodoacetate (which prevents lactic acid formation by inhibiting glyceraldehyde phosphate dehydrogenase), creatine phosphate breakdown supplies the energy for contraction; he went on to suggest that this might be the normal rôle of creatine phosphate hydrolysis in unpoisoned muscle also, the rôle of carbohydrate breakdown being to supply energy for resynthesis of creatine phosphate. In further experiments on contractions of very short duration or of a lightly loaded muscle, he showed that in these circumstances lactic acid formation was less in proportion to tension than under conditions of greater total tension production. These results, taken together with Nachmansohn's (1928, 1929) already mentioned, show that although the heat/tension ratio remains constant, the chemical reactions responsible for the heat production vary with time in a contraction series (Lundsgaard, 1931).

With regard to the anaerobic recovery, Lehnartz (1931) brought convincing evidence that much of the lactic acid production takes place after the contraction is over. Such claims made earlier by the Embden school had been disregarded, since with the strength of direct stimuli used a pathological condition of some fibers supervened, with prolonged relaxation time and incomplete recovery. Lehnartz now used stimulation through the nerve and established that 20 to 30% more lactic acid was formed in the 5 min. after relaxation. Indeed, at low temperatures, more than half the lactic acid formation may take place after relaxation (Meyerhof, 1931). In this lactic acid production, we have the energy source for the anaerobic resynthesis of creatine phosphate.

It is interesting that several other phosphagens have been found, all guanidino compounds; it is very probable that others still unknown

exist. Creatine phosphate is found in all vertebrate muscle examined (Eggleton and Eggleton, 1929-1930) and also in some invertebrates (Needham *et al.*, 1932; Baldwin and Yudkin, 1950). Arginine phosphate is the most widely distributed of the rest, being characteristic of most invertebrate muscle (Meyerhof and Lohmann, 1928a, b). Recently, however, the discovery has been made that certain invertebrates contain, as well as creatine phosphate, new phosphagens; thus in the annelids, glycocyamine phosphate has been identified in *Nereis diversicola* and taurocyamine phosphate in *Arenicola* (Thoai *et al.*, 1953; Hobson and Rees, 1955). Hobson and Rees (1957) have shown that in these animals phosphokinases are present which can bring about the phosphorylation of the bases in question by means of ATP. Thoai and Robin (1954) have isolated guanidylethylserylphosphate from the earthworm; chromatographic examination showed the presence of the corresponding phosphagen in its muscle. From leech muscle, a new hitherto unidentified guanidino compound has been isolated by Robin *et al.* (1957); since it is the only guanidino compound present, it is likely that it functions as a phosphagen. In each phosphagen, one hydrogen of the terminal amino group is replaced by the phosphate group.

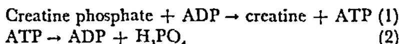
Creatine	$H_2N. C(:NH). N(CH_3). CH_2. COOH$
Arginine	$H_2N. C(:NH). NH. (CH_2)_3. CH (NH_2). COOH$
Glycocyamine	$H_2N. C(:NH). NH. CH_2. COOH$
Taurocyamine	$H_2N. C(:NH). NH. CH_2 CH_2. SO_3H$
Guanidylethylserylphosphate	$H_2N. C(:NH). NH. (CH_2)_2. O. (O)P(OH). O. CH_2. CH(NH_2). COOH$

C. The Discovery of Adenosine Triphosphate

Soon after the isolation of phosphagen there was discovered in muscle, independently by Lohmann (1929, 1931) and by Fiske and Subbarow (1929b), the substance adenosine triphosphate (ATP). Lohmann's studies (1932, 1935) indicated the structure shown in Table II; this has been confirmed by its synthesis (Baddiley *et al.*, 1948). Two observations of importance were made; in the first place, that ATP acted as a coenzyme of glycolysis, (Lohmann, 1929) though the details of its participation were not worked out till later; in the second place, that hydrolysis of the two terminal phosphate groups led to liberation of heat—about 12,000 cal. per gram molecule of phosphate, according to Meyerhof and Lohmann (1932).

The realization of the importance of ATP hydrolysis for the contraction process itself came only later, and arose out of the work of

Lohmann (1934) on hydrolysis of creatine phosphate in dialyzed cell-free muscle extracts. Such extracts cannot cause splitting off of phosphate from creatine phosphate; this only happens if adenylic compounds are present and the reaction occurs in two stages.



$$\text{Creatine phosphate} + \text{ADP} \rightarrow \text{creatine} + \text{ATP} \quad (1)$$
$$\text{ATP} \rightarrow \text{ADP} + H_3PO_4 \quad (2)$$

The first is a transfer of phosphate to adenosine diphosphate (ADP), then hydrolysis of the ATP thus formed goes on to give ADP again. It should be mentioned here in parenthesis that, in all the earlier work, adenosine monophosphate (AMP) was used in reaction systems which, as we now know, require ADP. The adequacy of the AMP is explained by the presence in such systems of the enzyme myokinase which enabled the AMP to react with traces of ATP.



$$\text{ATP} + \text{AMP} \rightarrow 2\ \text{ADP}$$

This work of Lohmann had consequences of great significance. Thus he deduced that before creatine phosphate breakdown can yield energy, ATP hydrolysis must have occurred; this latter reaction thus became the energy-yielding reaction closest to contraction. Further, this was the first observation of phosphate transfer, and involved two compounds each containing what we now call an "energy-rich phosphate bond" (Lipmann, 1941). Transfer of phosphate between such molecules without formation of inorganic phosphate is a mechanism for conservation of free energy which has turned out to be of enormous biological importance. At the time, Lohmann pointed out that the phosphate transport in reaction (1) (Lohmann's reaction) went on with very little heat exchange; he expressly remained noncommittal about free energy changes.

To provide for resynthesis of ATP, then, was the rôle of creatine phosphate in muscle metabolism. Parnas (Parnas *et al.*, 1934) next initiated an enquiry into the mechanism whereby carbohydrate breakdown provided energy and phosphate for the rephosphorylation, perhaps of creatine, perhaps of adenosine diphosphate.

It was known that hydrolysis of phosphopyruvic acid was an exothermic reaction (about 9,000 cal. being liberated per gram molecule of phosphoric acid released) and this seemed a likely stage; it was in fact found (Ostern *et al.*, 1935; Needham and van Heyningen, 1935; Meyerhof and Lehmann, 1935) in muscle extracts that phosphopyru-

vate (like creatine phosphate) transferred phosphate to adenylic compounds and was not dephosphorylated when these were absent.


Phosphopyruvate + ADP → pyruvate + ATP

In the presence of creatine as well as a catalytic amount of ADP, creatine phosphate synthesis took place, but no direct reaction between phosphopyruvate and creatine was found. It follows that reaction (1) must be reversible, and this reversibility has been directly demonstrated (Lehmann, 1936). The equilibrium point depends on the pH, more alkaline reactions favoring creatine phosphate synthesis. It seems then that in the recovering muscle, once the stimulation to ATP breakdown has ceased, the phosphate from carbohydrate intermediates is transferred through ATP mediation to free creatine to rebuild the creatine phosphate store.

Now Lundsgaard (1931) had found that for every molecule of lactic acid formed in the anaerobic recovery period, about two molecules of creatine phosphate were resynthesized. The reaction we have discussed could account for only half this resynthesis. But there is another exothermic reaction going on in glycolysis, the oxidoreduction between glyceraldehyde phosphate and pyruvate, giving as end products phosphoglyceric and lactic acids. This was known to be accompanied by esterification of inorganic phosphate (Meyerhof and Kiessling, 1935); it was now found that, if adenylic acid was added to the oxidoreduction system, stoichiometric synthesis of ATP went on, one molecule of phosphate being esterified for every molecule of lactic acid produced (Needham and Pillai, 1937; Meyerhof *et al.*, 1937). When this reaction also is taken into account, the extent of creatine phosphate synthesis in anaerobic recovery is readily understood. Although it has been known for some years that 1,3-diphosphoglyceric acid is formed in this coupled esterification and acts as the phosphate donor to ADP (Warburg and Christian, 1939; Negelein and Brömel, 1939), the sequence of reactions has only recently been made clear (Racker and Krimsky, 1952; Segal and Boyer, 1953). It involves reaction of the aldehyde group of the glyceraldehyde phosphate with the SH group of the enzyme, glyceraldehyde phosphate dehydrogenase; the oxidation of this hemimercaptal with formation of an "energy-rich" bond; and then phosphorolysis of the enzyme-acyl compound by means of inorganic phosphate to form diphosphoglyceric acid, which can transfer its acyl phosphate to ADP.

It is of interest to notice that in the case of 1,3-diphosphoglyceric acid, a direct transfer of the acyl phosphate to free creatine has recently been demonstrated in preparations from rabbit muscle, the intermediation of adenylic compounds being excluded (Cori *et al.*, 1956). The phosphokinase involving transfer to ADP was also present and of greater activity. The direct transfer system to creatine was apparently absent from the heart, a tissue depending primarily on aerobic metabolism.

The studies made by Meyerhof and his colleagues (Meyerhof and Lohmann, 1932; Meyerhof and Schulz, 1935; Meyerhof *et al.*, 1938) on the heats of hydrolysis of compounds involved in muscle metabolism led to the early distinction between compounds containing the guanidino-, pyro-, or enolphosphate linkage on the one hand, and the phosphate ester linkage on the other. Hydrolysis of the last was found to liberate only about 3,000 cal. per gram molecule of phosphate. It should be noted here that the heat of hydrolysis of ATP has been re-evaluated by several workers during recent years and considerably reduced. The latest value (found by enzymatic attack *in vitro*, in buffers of known heats of ionization) takes into account the heat of neutralization of H^+ ions produced during the reaction and amounts to only 4,700 cal. per gram molecule of H_3PO_4 set free (Podolsky and Morales, 1956). Redetermination of the values for the other heats of hydrolysis mentioned above is no doubt necessary; they have been quoted here because of their historical importance in the development of ideas on energy provision. The important aspect of these reactions is, of course the free energy and not the heat change; this was realized at the time, but since methods were not then available for measuring the free energies, the heats of reactions were taken as a rough guide. Since the treatment of this subject by Lipmann (1941), much effort by many workers has been put into the important task of finding true values for the free energy of hydrolysis of these compounds (Volume II, Chapter VII).

We have then this picture of the sequence of events after the stimulus reaches the muscle—first, dephosphorylation of ATP which, though masked by resynthesis in moderate contraction, is usually considered to be the essential reaction. As we shall see, there is good reason for postulating a direct reaction between the ATP and the myofibrillar protein actomyosin. Indeed, the ATP may break down in this initial reaction by a transfer of phosphate to some protein site. This is followed at once by reaction between creatine phosphate and ADP; later, re-

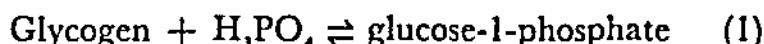
phosphorylation of ADP by phosphopyruvate and diphosphoglycerate from carbohydrate breakdown becomes quantitatively more important.

Attention has been concentrated here on contraction under anaerobic conditions, depending ultimately on glycolysis; this is because the reactions concerned, unlike many oxidative reactions, can go on readily in cell-free extracts, and so gave the first insight into problems of energy provision. But although anaerobic contraction must sometimes happen *in vivo*, conditions are much more usually aerobic, and oxidative rephosphorylation of ADP is far more efficient. This is discussed in Volume II, Chapter III.

D. Control of the Metabolic Rate on Stimulation

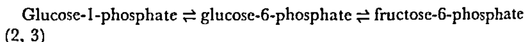
The presence of ADP may be considered as an adequate trigger, at pH values around 7, for the breakdown of creatine phosphate; its importance in regulating oxidative activity will be considered in Chapter 3. But it is not so clear what mechanism starts the rapid breakdown of glycogen which begins on stimulation. The adenine nucleotides are not involved in the earliest stages of glycogenolysis and adequate inorganic phosphate to saturate phosphorylase (the enzyme phosphorylating glycogen) is already present in the resting muscle. C. F. Cori (1956) has emphasized the enormous increment in rate of glycogenolysis following stimulation. For example, with frog muscle in nitrogen at 20°C., ten contractions per minute cause a thirtyfold increase over the resting rate and a 10 sec. tetanus causes a thousandfold increase. Now the enzyme phosphorylase exists in two forms, known as the *a* and *b* forms, the former active and the latter inactive without adenylic acid (G. T. Cori and Green, 1943). An enzyme is present (Cori and Cori, 1945; Keller and G. T. Cori, 1953) which brings about inactivation of phosphorylase *a*: the enzyme molecule is split giving a product of half the molecular weight, phosphorylase *b*; at the same time inorganic phosphate is liberated (Fischer *et al.* 1957). Fischer and Krebs (1955) showed also the presence in muscle extracts of an enzyme activating phosphorylase *b* in presence of ATP and Mg^{++} by transfer of phosphate to the enzyme. Cori (1956) believes both enzymes, the phosphorylase-rupturing and the phosphorylase *b* kinase, to be concerned *in vivo* in regulation of carbohydrate metabolism. With stimulation of rat gastrocnemius under controlled conditions, increase in active phosphorylase was seen, while fatigue led to a decrease. During a recovery period of 10 to 20 min., the level of phosphorylase *a* rose again.

It is not at first apparent why the activation of phosphorylase should lead to increased glycogen breakdown, since an equilibrium reaction is involved:

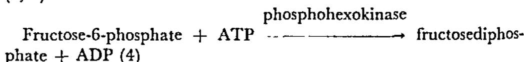


$$\text{Glycogen} + H_3PO_4 \rightleftharpoons \text{glucose-1-phosphate} \quad (1)$$

In order that there should be increased breakdown, increased rate of removal of hexosephosphate is probably also necessary. Indeed there is some evidence that the phosphohexokinase stage (see reaction 4 below) is limiting in resting muscle. Activation of this enzyme may result from the raised Mg/ATP ratio (Lardy and Parks, 1956) at some locations in the muscle cell as ATP is used in contraction.



Glucose-1-phosphate ⇄ glucose-6-phosphate ⇄ fructose-6-phosphate (2, 3)



Fructose-6-phosphate + ATP —phosphohexokinase→ fructosediphosphate + ADP (4)

II. INTERACTION OF ADENOSINE TRIPHOSPHATE AND ACTOMYOSIN AS THE BASIS OF MUSCLE CONTRACTION

A. The Early Experiments

During the years that saw this preoccupation with the energy sources of contraction, a parallel and quite independent line of work had been pursued in the study of the muscle proteins. As early as the middle of the nineteenth century, Kuhne had extracted from muscle a protein, capable of gel formation, which he called myosin. This protein was soon recognized as belonging to the class of globulins—soluble in NaCl or KCl solutions, but precipitated by dilution with large volumes of water (Weyl, 1877–1878). Edsall (1930) and von Muralt and Edsall (1930) purified the protein and studied its physicochemical properties, particularly the double refraction of flow of its solutions. Smith (1933–1934) investigated the dependence of the solubility of myosin on salt concentration and on pH. He concluded that under the conditions prevailing in resting muscle (pH about 7 and salt concentration equivalent to about 0.18 *M* KCl), at least 90% of the myosin must be in the gel form. About the same time, H. H. Weber (1935) was making myosin filaments by squirting the solution in 0.5 *M* KCl through fine extruders into a large volume of water, so that dilution precipitation took place.

It was not until some years later that Engelhardt and Ljubimova (1939) brought the two lines of work together by their discovery that myosin, prepared and purified by the classical methods, is a specific ATPase; when activated by certain divalent ions, it splits off the terminal phosphate group to give ADP.

This was very quickly followed by the finding that there is a reciprocal action of the substrate on the enzyme protein. Thus Needham *et al.* (1941; see also Dainty *et al.*, 1944) observed that addition of ATP to a

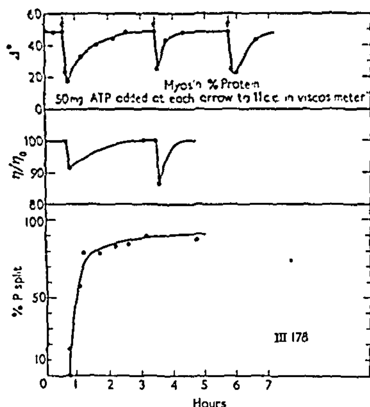


Fig. 1. Three successive falls and recoveries of flow-birefringence of a myosin sol treated three times with adenosine triphosphate, with two successive falls and recoveries of relative viscosity (expressed in percentage of initial value) and estimations of inorganic phosphate liberated by adenosinetriphosphatase activity of the myosin during the first cycle (Dainty *et al.*, 1944).

solution of myosin (made by many hours extraction of the muscle with salt solution) led to a striking fall in viscosity and double refraction of flow (see Fig. 1)—changes which are to be interpreted as a diminution in the axial ratio of anisometric molecules in the solution (Lawrence *et al.*, 1944). These changes were reversed as the ATP was destroyed by the enzymatic activity of the myosin. The tentative suggestion was made that a shortening of the myosin molecule took place and might be

the basis of contraction. But in Szent-Györgyi's laboratory in Hungary, where intensive study of these questions was also going on, it was shown that two proteins are concerned (see also Volume II, Chapter I): the protein which we now term myosin, extractable from minced muscle by short treatment (20 min.) with salt solution; and the protein actin, extractable from the dried residue (Banga and Szent-Györgyi, 1941–1942; Straub, 1942). Each of these proteins alone can give a solution of low viscosity and without double refraction of flow. On mixture of the two, a solution of high viscosity and strong double refraction of flow was obtained. The conclusion was drawn that the two proteins enter into some form of combination, giving actomyosin, and that the changes seen on adding ATP are due to dissociation of the complex, with formation of the two types of protein molecule of smaller axial ratio.

The relevance of the actomyosin-ATP relationship to contraction was more clearly brought out by the experiments of Szent-Györgyi (1941–1942) in which ATP was added to actomyosin at low ionic strengths, in fact to actomyosin gels. Actomyosin threads (prepared by Weber's method) were used and an isodimensional contraction to about 10% of the original length was obtained. If the micelles in the thread are oriented by partial drying and stretching (Buchthal *et al.*, 1947), the effect of added ATP is to make the thread become shorter and wider—as happens in contraction of a muscle fiber.

B. The Interaction of ATP and Actomyosin in Solutions

At the basis of most thinking on the contraction-relaxation mechanism, we find the conception outlined above—that addition of ATP to an actomyosin solution leads to dissociation of the two proteins, while removal of the ATP is followed by recombination. Bailey and Perry (1947) have given evidence for the view that the ATP displaces the actin, combining like the actin with sites on the myosin in the neighborhood of essential SH groups. Direct evidence for this dissociation conception was, however, for a long time lacking, but this gap has recently been filled. Thus A. Weber (1956) showed that if actomyosin is centrifuged (under conditions in which ATPase activity is inhibited) with ATP for 3 hours at 100,000 $\times g$, pure myosin could be recovered from the upper half of the supernatant and identified by its ATPase characteristics, its reaction with actin, and its sedimentation constant. The pellet on extraction yielded actin of characteristic behavior. Gergely (1956) has come to the same conclusion from light-scattering experi-

ments. The study of changes in light scattering of a system provides a rapid method of following changes in the size, shape, and interaction of the particles of the light-scattering material (see Oster, 1948). By application of the extrapolation method of Zimm (1948), particle weight can be determined independently of any assumptions as to particle shape. In this way, Gergely obtained evidence of a large fall in molecular weight upon ATP addition to actomyosin solutions, consistent with dissociation. The results with this method are, however, still the subject of controversy (see Section II A).

Mommaerts (1947) has shown that the viscosity drop on ATP addition can be obtained without any accompanying enzymatic dephosphorylation; the change is therefore due to combination of enzyme and substrate without breakdown of the substrate. Thus 10^{-3} *M* Mg^{++}, which inhibits ATPase activity of the actomyosin solution, allows the unimpaired viscosity fall, but there is no recovery. More recently, he has studied the same change in the protein particles by the more sensitive light-scattering method and has shown that Ca^{++}, while accelerating the ATPase activity, greatly decreases the light-scattering fall (Mommaerts, 1956). The high rate of ATP disappearance may have been partly responsible for the diminished fall; but the results of Baranyi *et al.* (1951), using a method of very rapid measurement of

TABLE I

THE INFLUENCE OF MAGNESIUM AND CALCIUM ON THE RATE OF VISCOSITY FALL WHEN ADENOSINE TRIPHOSPHATE IS ADDED TO ACTOMYOSIN SOLUTIONS[a]

(Results expressed as half-time of the fall in seconds)

No activator	Mg^{++}			Ca^{++}			0.004 *M* Mg^{++} plus 0.003 *M* Ca^{++}
	0.001 *M*	0.01 *M*	0.004 *M*	0.001 *M*	0.002 *M*	0.004 *M*	
9.5	0.63	0.47	0.54				
6.0				9.5	7.9	3.0	
							0.66

[a] The relative viscosity of the actomyosin solution was 2.5; the ATP concentration 0.5×10^{-4} (Baranyi *et al.*, 1951).

viscosity changes, show that there really is a very marked activating effect of Mg^{++} ions and a smaller inhibitory effect of Ca^{++} ions on combination between actomyosin and ATP (see Table I). This point will be raised again later.

Straub (1943) and Mommaerts (1947) further showed that inorganic pyrophosphate which is not hydrolyzed (Bailey, 1942) and inorganic triphosphate which is only very slowly hydrolyzed (Dainty *et al.*, 1944) can in certain circumstances show the unreversed viscosity fall. A high concentration of Mg^{++} (0.01 *M*) is necessary here and Ca^{++} is ineffective.

C. The Interaction of ATP and Actomyosin in Gels

1. *The Types of System Used*

We have already mentioned the use of the actomyosin thread after orientation by partial drying in a stretched state, as a model of the muscle fiber. An even more useful preparation, introduced by Szent-Györgyi (1949), is the glycerinated fiber bundle. A strip of muscle about 2 mm. in diameter is removed, being kept at the resting length; from the psoas of the rabbit, for example, a bundle of parallel fibers some 8 cm. long can easily be obtained. The fiber bundles are kept in 50% glycerol at 0°C. for some days. In this way, the removal of water from the muscle is very gradual and the water content remains uniform throughout the bundle; about 50% of the soluble proteins and most of the crystalloids are extracted. An important characteristic is that, owing to destruction of membranes, the fibers show no response to electrical stimulation but are permeable to ATP. Such fiber bundles can be kept for many weeks; they can be washed free from glycerol when needed, dissected to smaller dimensions, and then used for the study of tension production upon addition of ATP. The tension production and degree of shortening are quantitatively very similar to the responses of living muscle to electrical stimulation (Weber and Portzehl, 1952).

2. *The Stages in the Interaction and the Dual Rôle of ATP*

The first investigation of the effect of ATP on such muscle models was made by Engelhardt *et al.* (1941) on actomyosin threads. These contained only 2% of protein but showed a considerable amount of tensile strength. When a load of some milligrams was applied by means of a torsion balance to the thread immersed in a bath, extensibility could be measured. Addition of ATP to the bath led to an *increase* in extensibility by some 50 to 100%. At first sight, these results seem to be in contradiction to those of Szent-Gyorgyi, made a little later, in which as we have seen, the effect of ATP was to cause contraction. Some years later, Buchthal *et al.* (1947) showed that the same threads (in this case

20% protein) would give both effects—if loaded, e.g. with 200 mg., they showed extension on ATP application; but unloaded, they contracted. By very carefully controlled drying and stretching, it is possible to prepare actomyosin threads which will develop considerable tension (Portzehl, 1951), but the point made here is that the extensibility increase with ATP indicates that the first effect on the gel is a loosening of linkages, as with the solution. There is much evidence, as we have seen, that in the case of the solution, myosin and actin are dissociated

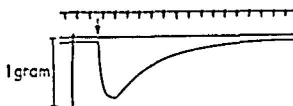


FIG. 2. Brief contraction of glycerinated fiber bundle produced by 1.5% ATP (about 0.03 *M*). Temperature, 21°C. Time marks every 5 sec. Tension = 1 g. (Bozler, 1951).

from one another; while the same degree of dissociation cannot take place in the gel, it is quite possible that the same bonds are affected. Szent-Györgyi (1949), using glycerol-extracted muscle fibers, emphasized this dual rôle of ATP. After treatment with ATP the contracted fiber bundle was hard and opaque, but on renewal of the ATP it became momentarily soft and flexible before hardening again as the fresh ATP was used up. Bozler (1951, see Fig. 2) was the first to get a full cycle of contraction and relaxation; here, with the glycerinated fiber bundle, a high ATP concentration was used (0.02 *M*) and the contraction was brief. A concentration of 0.003 *M* ATP caused only contraction.

A better understanding of the dual rôle of ATP in muscle began with the work of Marsh (1952). He used fresh muscle homogenates, in which the fragments consisted of fiber bundles, and he followed their changes in size by centrifuging and measuring the volume of the solid layer. When a brei from fresh, actively glycolyzing muscle was used, the fiber volume remained constant for a considerable period, depending upon the glycogen content of the muscle. After 20 to 50 min., there was a rapid fall in volume, then again a steady state. He considered that the fiber shrinkage corresponded to contraction, the water loss which occurred from the fibers being a consequence and not the cause of the diminution in volume. Experiments in which the behavior of the fiber fragments was examined under the microscope bore out this point of view. With fresh homogenates, ATP addition usually led first to an increase in volume and

length of the fibers (relaxation); only later, after a time during which diminution of the ATP concentration within the fibers (through their own low ATPase activity and that of soluble ATPase) would have occurred, did the fibers shorten. The rise and fall in volume could be repeated several times.

Marsh was led to emphasize the importance of ATPase activity by the observation that if the fibers were washed twice with salt solution, the only response to ATP added to the suspension was decrease in volume, never increase. At the same time, the ATPase activity of the fibers rose some tenfold as a result of the washing. Marsh deduced the presence in the original brei of a relaxing factor responsible for these effects and we shall return to this later. He also deduced that dissociated actin and myosin in the relaxed muscle must change to actomyosin before contraction; for this change and the shortening, both an increased rate of energy liberation from ATP and a fall of ATP concentration at the active sites on the protein are necessary. He did indeed find that the contraction only occurred when the ATP concentration within the fibers had fallen (Marsh, 1951).

Thus while earlier work on the effects of ATP on gels simply laid emphasis on the shortening response upon application of the nucleotide, later the question of the correlation of the effect with ATPase activity was investigated. We shall discuss this further. The system used by Marsh was too complex to allow proof of his deductions, and there has been much further work on these questions. Meanwhile we may anticipate the point of view which will be developed in what follows by considering in stages the action of ATP on actomyosin gels *in vitro*, as we have already done for its action on actomyosin solutions.

First we have the loosening of linkages. Second, ATPase activity supplies energy and reduces the ATP concentration at the active sites. Third, the formation of new linkages bringing about the shortening is now possible. From all the evidence of Section I, we must conclude that continuous ATP breakdown is necessary as long as tension production goes on. ATPase activity will continue until all the ATP is used up, and the fiber then remains in the shortened form. Under certain conditions, fresh ATP can cause relaxation.

3. *Problems of Diffusion*

In experiments such as those of Marsh, the fresh fiber fragments can for a time keep up their own internal ATP concentration by carbohydrate metabolism; with experiments with the artificial thread or the

glycerinated fiber, there is dependence from the beginning on the balance between the rate of diffusion inwards of the ATP on the one hand, and the rate of hydrolysis by the actomyosin on the other. In the steady state, the concentration of ATP on the outside (denoted by C) is related to the concentration at the center (denoted by I) according to the Meyerhof-Schulz formula (Meyerhof and Schulz, 1927):

$$C = Ar^2/4D + I$$

where A = rate of splitting, r = radius of the fiber, D = diffusion constant of ATP within the fiber.

Calculation shows that with a physiological concentration of ATP (about 5×10^{-3} M), the diameter of a fiber must not exceed about 6 μ if the concentration is to be the same in the center (Hasselbach and Weber, 1955). With a fiber bundle 500 μ in diameter, the concentration at the center would be zero. In such a case, the fibers of a large central core remain stiff and play no part in the contraction, indeed even hinder it (A. Weber, 1951). Tension measurements have been successfully carried out with thin single fibers, for example by Briggs and Portzehl (1957); here the fibers were 50 to 60 μ in diameter. They showed a much higher tension production per square centimeter, about twice as great as with fiber bundles, presumably because the inactive core is much smaller. Even so, it was calculated that the ATP-free core would be 20 to 50% of the cross section; but it is much less likely than in a thicker fiber to be in a condition of rigor since it has available ample supply of ADP, a good plasticizer (cf. Engelhardt, 1946) in the presence of enough Mg^{++}. It will be obvious that correlations of tension production and ATPase activity will present difficulties when fiber bundles are used, since the exact conditions of diffusion will vary from one bundle to another.

When on the other hand, comminuted glycerinated fibers or isolated fresh myofibrils some 2 to 3 μ in diameter are used, there is no diffusion problem, and enzymatic activity under different conditions can be accurately studied. Correlation of this ATPase activity with rate or degree of shortening is sometimes usefully made, but it is impossible to assess how far these mechanical effects can be taken as an indication of the power to produce tension.

D. ATPase Activity of Myosin and Actomyosin

Purified myosin, free from actin, has ATPase activity, but actin has none. It is therefore assumed that the ATPase activity of actomyosin,

though it shows some characteristic differences (for references, see Needham, 1952), is due to sites on the myosin component. An important difference between actomyosin and myosin ATPase is that the former is activated by Mg^{++}, the latter not (Banga, 1941–1942). This Mg^{++}-activation is seen with actomyosin at low ionic strength; above about 0.2, addition of ATP leads to dissociation so that then the enzymatic characteristics of myosin ATPase appear (Hasselbach, 1952). Both ATPases are activated by Ca^{++}.

In view of the various indications that Ca^{++} and Mg^{++} ions are concerned with different sites on the myosin molecule, it is of interest to notice that the activation energy of the Mg^{++}-activated ATPase is much greater than that of the Ca^{++}-activated. Bendall (personal communication) has found an increase in rate of some 400-fold with the former on passing from 0°C. to 35°C.; with the latter, the increase was only some 20-fold. Hasselbach (1952) and Perry and Chappell (1957) have also noticed the high temperature coefficient of Mg^{++}-activated actomyosin.

The ATPase activity of these two proteins has some special features which may be of importance, as we shall see, in contraction and relaxation.

1. *The High Initial Rate of ATPase Activity*

Weber and Hasselbach (1954), using glycerinated fibers 30 μ thick, showed that the ATPase activity was at least twice as great during the first 15 sec. of reaction at room temperature as later—after about 100 sec., when a constant rate was reached. Accumulation of end products, decreasing concentration of ATP, impurity in the ATP, and contraction of the originally relaxed fibers were all considered ruled out as causes of the decrease in rate. The effect could be repeated if the hydrolysis was interrupted by Salyrgan inhibition, then reactivated by cysteine; it was in fact obtained only with fibers newly beginning to cause splitting. The authors suggest that there may be slow reversible formation of an inactive enzyme-substrate complex of the sort described by Chance (1948) with catalase. The experiments described were done with Mg^{++} activation, though a case is given where Ca^{++}-activated myosin gave a similar but smaller effect. Bendall (personal communication) has found the effect to be consistently much smaller with Ca^{++} than with Mg^{++}-activation of myofibrils.

2. *Phosphorylation of the Enzyme*

On analogy with its other important biochemical reactions (see Section I), it has often been suggested that, in ATP breakdown, the

first stage is transfer of its terminal phosphate to some site on the myosin—or perhaps on the actin. If this mechanism does indeed operate, one should be able to find exchange between ADP[32] and ATP in presence of the enzyme. Koshland *et al.* (1954) were unable to find such a reaction catalyzed by Ca^{++}-activated myosin or actomyosin. Thus if a phosphorylated protein intermediate is formed under these conditions, its existence must be transitory. This, as H. H. Weber (1955) has emphasized, is only to be expected, since a virtually irreversible, energy-yielding reaction would probably follow at once; moreover, any labeled ATP formed by the back reaction would be in a more favorable position for dephosphorylation than incoming ATP, since the former would be already in the neighborhood of the active sites.

Ulbrecht and Ulbrecht (1957), using highly purified natural actomyosin or isolated myofibrils washed very thoroughly, showed that an exchange could readily be detected provided that Mg^{++} was the activator. However, this exchange was found to be independent of the presence of myosin—it continued in myofibril preparations from which the myosin had been removed. It is difficult to conclude that this means actin phosphorylation, since purified actin showed no exchange, although of course it can combine with myosin to give an actomyosin of normal ATPase activity and normal contractility in the gel form. The situation thus remains obscure.

3. *Substrate Inhibition*

The existence under certain conditions of an optimal concentration of ATP for the ATPase activity of actomyosin has been realized since the work of Weber and Weber (1951). They used glycerinated fibers and the phenomenon was further studied by Hasselbach and Weber (1953). Recently, Perry and Grey (1956) have turned attention to this effect of overoptimal ATP concentration in connection with the mechanism of relaxation.

Using washed fresh myofibrils and working in the range of 2.5 to 10×10^{-3} *M* for both ATP and Mg^{++}, they found that the ATP became inhibitory when its molar concentration exceeded that of the Mg^{++}. This relationship depended to some extent on the previous history of the myofibrils; it is well seen with myofibrils freshly isolated in 0.1 *M* KCl, and tested in a medium of about physiological ionic strength, 0.16. It does not, however, hold at lower concentrations of the reactants. Thus Geske *et al.* (1957) have found that with Mg^{++}

concentrations below 5×10^{-4} *M*, the overoptimal ATP concentration shifts (with increasing Mg^{++}) first to *lower* ATP concentrations.

Perry and Grey found that marked substrate inhibition is characteristic of Mg^{++}-activated ATPase; it is very slight with Ca^{++} as activator. Very low Ca^{++} concentrations can abolish the substrate inhibition of the Mg^{++} activated system.

E. Contraction and ATPase Activity *in vitro*

Weber and his collaborators have brought forward much evidence showing the close correlation between tension production in glycerinated fibers and their ability to dephosphorylate ATP. Thus Weber and Weber (1951; Heinz and Holton, 1952; see also H. H. Weber, 1951) showed the effect of changing ATP concentration upon both tension and ATPase activity; the changes ran parallel both at room temperature and at 0°C. Ulbrecht and Ulbrecht (1953), with glycerinated fibers from smooth adductor muscles of *Anodonta*, found that the mechanical effects of ATP application and the ATPase activity showed the same temperature dependence. Bendall (1953a), working on the relaxing factor, found that as his preparations decreased the rate of shortening with glycerinated fiber fundles, so also they caused lowered ATPase activity. In all these cases, Mg^{++} was used as the ATPase activator and was supplied in the medium of the contracting fibers.

Bowen and his collaborators, on the other hand, have described a number of cases which they consider can better be explained by correlating combination of ATP and actomyosin (rather than ATPase activity) with contraction. Thus they found conditions in which addition of Mg^{++} ions accelerated shortening of actomyosin filaments in presence of ATP, while homogenized preparations from the same material showed unchanged or decreased ATPase activity. On the other hand, Ca^{++} ions, which increased the enzymatic activity by a factor of 4, decreased the rate of shortening (Bowen, 1952). At this point, it is important to remember that the phenomenon of contraction is complex: ATP splitting may be an essential feature, but those who take this point of view would not regard it as the only essential requirement. Thus there seems good reason to believe that Mg^{++} ions play an important role (which cannot be filled by Ca^{++} ions) both in viscosity and in gelation changes (see Sections II, B and VI); that they should play a specific part in the shortening process is therefore not unexpected. Indeed, Bendall (personal communication) has found, with well-

washed glycerinated fiber bundles (about 200 μ in diameter) shortening under load, that there was virtually no work performance in 6 m*M* ATP when Ca^{++} was added up to 8 m*M*, although this Ca^{++} concentration can stimulate about maximal ATPase activity. Addition of 0.4 m*M* Mg^{++} to the bath (with or without Ca^{++}) led to good work production and ATPase activity. It seems, then, that the correlation is between Mg^{++}-activated ATPase activity and contraction. See also Fig. 5, showing Mg^{++}-activation of contraction with inosine triphosphate, Ca^{++} in the same concentration having no effect (Watanabe and Sleator, 1957).

Again, increasing the KCl concentration in the presence of Mg^{++} ions leads to accelerated shortening, while the enzymatic activity is depressed (Bowen and Kerwin, 1955). Here, as Perry (1956) has pointed out, the increased ionic strength may, by reducing hydration of the fibers, improve their mechanical properties, quite independently of any effect on enzyme activity.

Further, it has been found that the final degree of shortening of glycerinated fiber bundles varied with the concentration of ATP supplied, reaching in each case a constant value (Blum *et al.*, 1957). The conclusion was drawn that the degree of shortening did not depend on the amount of ATP split, but on the concentration present, i.e. on the amount bound to the protein. But in these circumstances, the limiting factor in the hydrolysis rate in the interior of the fibers would be the rate of diffusion, itself dependent on the concentration. There is nothing in these data to show that the shortening did not depend on the splitting rate; this is admitted (Bowen, 1957) but it is maintained that the comparison of the effects of Mg^{++} ions with those of Ca^{++} ions rules out this possibility. However, we have already shown the reasons for regarding this last argument as unsatisfactory.

Many reagents reacting with SH groups are known to inhibit the ATPase activity of myosin. Their effect on contraction of actomyosin threads or fibers has always been found to be similarly or even more strongly inhibitory (Turba and Kuschinsky, 1952; Weber and Portzehl, 1954). In some cases (Portzehl, 1954), the ATPase activity of fibers after partial poisoning may be higher at room temperature than that of the unpoisoned fiber at 0°C.; yet the latter will contract, the former not (cf. Hasselbach and Weber, 1955). It would seem that, in the former, the energy released is not available for contraction. It may be that SH groups are essential for reaction along the protein chain as well as for ATPase activity.

To sum up, we may say that the evidence strongly favors the necessity of participation of Mg^{++}-activated ATPase in contraction; but the contraction process is complicated, involving more than this enzymatic activity.

III. MECHANISM OF THE ACTOMYOSIN ADENOSINE TRIPHOSPHATE INTERACTION IN CONTRACTION

We must now consider the stages of this interaction in more detail in relation to the mechanism of contraction. Ever since the realization of the part played by ATP, opinion has been divided as to whether its phosphate was liberated during the contraction or the relaxation phase; we shall deal with arguments put forward on both sides.

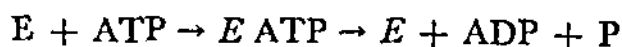
It must be remembered that, until a few years ago, the view was generally held that contraction was produced by the folding of protein chains which ran throughout the muscle length. Recently, much evidence has accumulated that striated muscle contains two types of filament and that the individual filaments do not change in length as the muscle shortens. From these observations, the idea follows that the two types of filament slide over one another to produce the shortening. It has been suggested that a cyclic mechanism underlies this relative movement, linkages between the two types of filament (actin and myosin) being made and broken during contraction as the filaments pass each other (Hanson and Huxley, 1955; A. F. Huxley, 1957). Such a mechanism will be considered in Section III, C.

A. Theories Involving the Liberation of Free Energy by ATP Hydrolysis during Relaxation

Szent-Györgyi has for many years been a strong protagonist of this point of view (Szent-Györgyi, 1953), though it would seem that he has recently joined those who regard the contraction phase as the one needing direct provision of energy by ATP breakdown (Szent-Gyorgyi, 1956). He had supposed that upon stimulation, actin plus myosin forms actomyosin, and that ATP then combines with the myosin. In doing so, it enables a new endergonic link to be formed at the expense of the energy-rich phosphate bond. The establishment of this link has made certain joints pliable and these now fold, as a result of electrostatic attractions and repulsions along the chain. In order that relaxation may take place, the new energy-rich link must be broken, and this is done by the removal of ATP from the protein as ADP + H_3PO_4.

A variation of this view is that of Riseman and Kirkwood (1948); they postulated the phosphorylation of OH groups along the protein chain, which was then held extended by repulsion of the negative charges. On stimulation, dephosphorylation took place, the charges were thus abolished, and the chain was free to contract. In relaxation, rephosphorylation of OH groups by ATP was necessary. On this theory, inorganic phosphate would actually be released during contraction, but the loss of free energy by the breakdown of ATP and formation of the phosphate ester linkage would occur during relaxation.

Next we come to the views of Morales and his collaborators, put forward during the last few years and summarized by Morales *et al.* (1955). These studies have been made on lightscattering by solutions of myosin B, i.e. the protein obtained by 5 to 24 hours' extraction, and considered by most workers to consist of a mixture of actomyosin and myosin, the former in much greater amount (see Dubuisson, 1950). Morales and his co-workers find the evidence for the presence of actin inconclusive, and consider the lightscattering phenomena to be due to myosin alone. The lightscattering method, as we have seen, is a very sensitive and rapid one for following changes in shape and size of protein particles. The effect of added ATP is a fall in the light scattering which persists for a time (during dephosphorylation of the ATP) and then there is a return to the original value. Below a certain ATP concentration, the degree of change depends on the concentration of the ATP added. Further when the experiment was carried out in presence of 0.001 *M* Mg^{++} (which inhibits dephosphorylation at this high ionic strength), the lightscattering change (like the corresponding viscosity change) was unaffected in degree, but now was not reversed. This was interpreted to mean that the deformation of the particles depended on their combination with ATP and went on irrespective of ATP breakdown.



$$E + ATP \rightarrow \mathit{E}\,ATP \rightarrow \mathit{E} + ADP + P$$

where *E* represents the enzyme after deformation.

By studying the reaction at different temperatures, Morales and his collaborators (Blum and Morales, 1953) consider that they have evidence that the light-scattering changes can go on without a change in molecular weight of the particles—i.e. without dissociation. In this, although using the same Zimm method, they are at variance with Gergely (1956; Gergely and Kohler, 1957). In later work (von Hippel *et al.*, 1957) evidence for some dissociation was found by the Morales

group, but the authors consider that only different states of aggregation of myosin itself were concerned when this happened. Gergely and Martonosi (1958) on the other hand, have obtained further evidence in support of their interpretation, that dissociation of actomyosin is concerned, by preparing typical actin from 3 to 6 times precipitated myosin B. Morales and his collaborators (Ouellet *et al.* 1952) concluded that the combination between protein and ATP is an energy-providing reaction ($\Delta F^\circ = -6600$ cal. per mole). They consider this deformation of the protein particles to be responsible for contraction and to depend upon the charge. They picture the degree of deformation as governed by a conflict between extensile electrostatic forces and contractile entropic forces—the particles gain configurational entropy on shortening, whether the shortening is by random coiling or by folding into an ordered form by reaction between certain groups. For relaxation, an energy-providing reaction is needed, to overcome the electrostatic effects of the bound ATP, e.g. the removal of the ATP from the protein by enzymatic breakdown to ADP and inorganic phosphate (Morales and Botts, 1956). The experiments that we have already described in which ATPase activity and contraction could be to some extent affected independently are cited as evidence for this point of view.

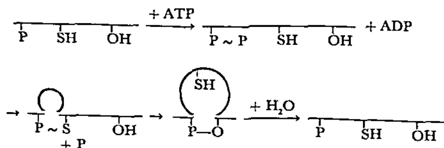
There is indeed abundant evidence that combination between ATP and myofibrillar protein in solution takes place and that "deformation" of the protein occurs before ATP dephosphorylation. Other workers (Tonomura *et al.*, 1953) have obtained results by the light-scattering method similar in many respects to those just described. The experiments employing the viscosimetric method cited earlier in Section II, B indicate the same thing. However, there seems to be, as we found in considering the effect of ATP on actomyosin solutions, good evidence that the "deformation" is a dissociation of myosin from actin. When the effects upon a sol were compared with those on various forms of gel, we regarded the initial increase in extensibility as a comparable dissociation, a preliminary loosening of linkages putting the rigid gel into a fit state for contraction. In the resting muscle, as we shall shortly see, this step is unlikely to be necessary, since electron micrographs seem to show the actin and myosin as distinct and separate filaments. But recent work has made it likely that any contraction involves a cyclic process of linkage-making and linkage-breaking, so that this dissociation stage would be involved during the contraction phase (to make possible each further shortening step) and during relaxation.

B. Theories Involving the Direct Utilization during Contraction of the Free Energy of ATP Hydrolysis

We have already discussed a good deal of experimental work which, taken at its face value, would seem to support this point of view. However, it is obvious that no enzymatic action by the actomyosin upon the ATP can take place without previous combination of the two, so that there is a logical flaw in any argument from a correlation between tension production or shortening and ATPase activity. The most striking evidence in support of this standpoint—that the energy of ATP hydrolysis is needed in the contraction phase—is that brought forward by H. H. Weber (1951) and by Bozler (1951). We have already seen that a glycerinated fiber, made to contract in ATP solution, and remaining rigid and contracted when excess ATP is washed away, can be made to show a momentary relaxation by the addition of more ATP. Weber and his collaborators now showed that this relaxation can be brought about in circumstances allowing no simultaneous energy provision. Thus ATP can bring about complete and lasting relaxation in the presence of the mercurial Salyrgan, which prevents its hydrolysis by poisoning the actomyosin ATPase (Portzehl, 1952). Salyrgan alone has no effect on the fiber. Inorganic pyrophosphate (0.015 *M*) also gave the same effect, although it is not a substrate for actomyosin ATPase. Bozler also got relaxation of ATP-contracted glycerinated fibers when 0.02 *M* pyrophosphate was added, and concluded that the relaxation process involves no large energy change. Bendall (Bendall, 1953b) has got similar results with much lower pyrophosphate concentration (0.004 *M*), provided Mg^{++} (0.004 *M*) was also added.

This view of the timing of energy provision accords with A. V. Hill's finding (1949b) that the relaxation phase (provided the muscle relaxes unloaded) is free from heat production.

H. H. Weber (1955) has put forward a scheme which enables one to visualize possible mechanisms whereby the free energy of hydrolysis could be directly used in the contraction of the protein molecule.



Schemes such as this must inevitably be open to criticism as regards their details. Thus relaxation is pictured as brought about by hydrolysis of the ester linkage, and it is difficult to see how the relaxing effect of ATP fits in here. Morales and Botts (1956) have pointed out that the forces leading to establishment of covalent linkages are of very short range; further, such forces would become larger as the muscle shortened, while in fact the isometric tension developed becomes less as the muscle shortens. These latter objections are to some extent overcome when some such mechanism is envisaged (Weber, 1958) acting as part of a cyclic process (see Section III, C). The evidence concerning the possibility of phosphorylation has already been considered (see Section II, D, 2).

C. The Sliding Hypothesis, with a Cycle of ATP Breakdown and Resynthesis during Contraction

Electron micrographs of striated muscle have shown that two sets of filaments are present in regular hexagonal array and parallel to the long axis (H. E. Huxley, 1953a). Of these, the larger (diameter about 100 A.) have been identified as consisting mainly of myosin, the more slender (40 A. in diameter) as mainly of actin. The myosin filaments occupy the A band; the actin filaments run from the Z lines at the center of the I band and into the A band as far as the beginning of the H zone (H. E. Huxley and Hanson, 1954). Upon contraction (to 65–70% of the resting length), the A band remains of constant length, but the H zone and the I band shorten (A. F. Huxley and Niedergerke, 1954; H. E. Huxley and Hanson, 1954). These changes are best explained by a sliding of the actin filaments past the myosin into the A band, without folding of either type of filament unless shortening of the muscle is very great. The evidence from low-angle diffraction diagrams obtained with living muscle supports the conclusion that the filaments themselves do not change in length (H. E. Huxley, 1953b). The problem then is to discover the mechanism whereby the actin filaments are drawn past the myosin filaments. This subject of the fine structure of muscle is considered in Volume I, Chapter VII, and there hypotheses concerning this mechanism are discussed. Here we wish only to link up the problem with the biochemical findings.

In most of the suggested hypotheses, the contraction proceeds by stages. Sites on the actin filament can be pictured as oscillating by means of thermal agitation backwards and forwards past sites on the

myosin filament with which it overlaps. As a result of the stimulus, linkages are formed between the two sites and a certain degree of contraction occurs. When these links are broken by the arrival of fresh ATP, the actin sites are now within range of further myosin sites and the process is repeated further and further along the myosin chain. A schematic formulation is given below, based on what we know from studies *in vitro* of the interactions of actin, myosin and ATP.

(1) (During the period of active contraction)	M.ATP → M ~ P + ADP
(2) (Formation of links, shortening stages)	M ~ P + A → AM + P + free energy used as tension or work
(3) (Loosening of links, finally relaxation)	AM + ATP → M. ATP + A

Reaction (1). It is supposed that the effect of the stimulus is to cause a transphorylation which provides one of the proteins (myosin is suggested here but it might be actin) in a state ready to combine.

Reaction (2). The free energy of the energy-rich bond is used in some reaction or series of reactions leading to the formation of contracted actomyosin. This series of reactions might be, for instance, along the oblique connecting side chains suggested in one hypothesis (Hanson and Huxley, 1955) or occupy the place of the "spring" in the hypothesis of A. F. Huxley (1957). Formation of covalent bonds might be concerned as in Weber's formulation (see Section IIIB); electrostatic forces or hydrogen bonds might be concerned—we do not know.

Reaction (3). The loosening of the linkages would be due to the arrival at the sites of fresh ATP, by diffusion.

On this formulation, ATP is concerned during contraction with both making and breaking of links; during relaxation, only with breaking of links. There is no requirement for energy provision during relaxation. The activation heat of Hill (1949a, 1950) could arise (a) possibly in connection with unknown reactions between stimulation and reaction (1); (b) possibly from some ATP dephosphorylation in the neighborhood of the sites, necessary to reduce it below a critical concentration; (c) perhaps from some heat wastage in reaction (1); (d) from heat production connected with reaction (2), before enough links are formed to permit a mechanical response. The shortening heat (Hill, 1949a), only produced when the muscle actually shortens, would be derived from reaction (2), possibly also from reaction (3), though the latter

contribution would have to be small as no heat is detectable during relaxation without load.

A very striking aspect of muscle metabolism is the constancy of the heat of shortening for a given muscle. Whether the muscle contracts slowly under a load or rapidly unloaded, the extra heat associated with a given amount of shortening is the same. The energy necessary for the accomplishment of the work is specially mobilized in proportion to the load and the shortening heat is not diminished to provide any part of it (Hill, 1949c). Now as the muscle contracts more slowly when it is loaded, in these conditions more time is available and the same links might be formed and broken several extra times. We may surmise that this is the source of the extra energy which appears as work.

Bendall (1959) has recently drawn attention to an assumption which is necessary if the sliding hypothesis is to be reconciled, as it must be, with the proportionality between shortening heat and distance shortened. It seems that as the actin and myosin filaments slide over each other, the number of reacting groups per unit of shortening must remain the same. Two alternative possibilities present themselves: (a) on the actin filaments only the tips are reactive and groups situated here react with successive groups on the myosin; (b) on the myosin filaments only the ends near the I band bear active groups and these react with site after site on the actin filaments. In either case a biochemical problem is presented, since so far as is known each type of filament is homogeneous along its length. Bendall makes the tentative suggestion that tropomyosin, quantitatively a minor constituent of the myofibril (Perry and Corsi, 1958) may have the rôle of protecting some parts of the major proteins from interaction.

The effort to go deeply into the sliding mechanism is only just beginning. A. F. Huxley (1957) has suggested a very interesting picture, involving oscillation of the myosin sites about an equilibrium position in the neighborhood of the actin sites, with certain postulations about the rate constants of the making and breaking of links, and the factors affecting these constants. Very reasonable quantitative agreement was obtained between the expectations worked out mathematically from this hypothesis and a great number of the thermal and mechanical observations on living muscle recorded in the literature.

IV. ENERGY PROVISION IN THE LIVING MUSCLE

Using evidence from innumerable observations and experiments *in vitro*, we have built up the picture of an actomyosin-ATP machine

depending for its energy supply on ATP dephosphorylation. We wish now to inquire how well this picture fits the requirements set by phenomena *in vivo* as regards the arrangements for the fuel supply. That is to say, how good is the evidence that ATP breakdown is *in vivo* the primary energy-yielding reaction? It is not to be expected that, with a fresh, unfatigued muscle, such evidence will easily be produced, since, as we have seen, there are powerful enzyme systems in the muscle for rephosphorylation of ADP. Nevertheless, a number of lines of inquiry can be explored.

A. ATPase activity *in vivo*

In the first place, we may ask whether the activity of actomyosin ATPase under conditions *in vivo* (about pH 7 and ionic strength about 0.18) is high enough to account for the energy production if all this is channeled through ATP breakdown. It seems that we must consider the behavior of actomyosin ATPase, not that of free myosin ATPase. Muscle contains both Mg^{++} and Ca^{++}, but there is good evidence that only Mg^{++} activated actomyosin ATPase is concerned with contraction.

The results of Perry and Grey (1956) give a figure of about 0.3 μ mole per milligram protein per minute for the Mg^{++}-activated ATPase of well-washed rabbit myofibrils at 20°C. The figures of Hasselbach (1952) are similar for finely-divided actomyosin gel at 23°C. Thus a figure of 1 to 2 μ moles per milligram protein per minute might be expected at 37°C., or 1 to 2 $\times$ 120 μ moles per gram muscle. This assessment takes no account of the high initial rate of inorganic phosphate liberation (see Section II D), which may be much more than double the rate estimated by observation over several minutes. If this high rate is substantiated as due to true ATPase activity, we must suppose it would operate in the conditions of discontinuous ATPase activity we have been contemplating. With 120 mg. of actomyosin per gram of muscle, the rate would then be at least 2 to 4 $\times$ 120 μ moles, or 2.4 to 4.8 $\times$ 10^{-4} moles per gram muscle per minute. Bendall (personal communication) has indeed found initial values at 35°C. for rabbit myofibrils corresponding to 5–6 $\times$ 10^{-4} moles per gram muscle per minute. Mommaerts (1950) had calculated the utilization of energy-rich phosphate which would correspond to the maximum effort in human muscle; these calculations are based on the extra oxygen consumption, on the assumption that 5–6 energy-rich phosphate bonds are formed per molecule of oxygen used, and include corrections to allow for the actual amount of muscle tissue involved in the increased metabolism. It seems

that there may be a 500-fold rise in metabolism, and the metabolism of the energy-rich phosphate bond should reach 10×10^{-4} moles per minute per gram of muscle. The comparison of ATPase activity of rabbit muscle preparations with the muscle metabolism of man is open to criticism, since the small mammals show greater metabolic activity than the large. However, considering the roughness of all these calculations, the measure of agreement between the need and the supply is reassuring.

B. pH Changes in Living Muscle

In experiments of Dubuisson (1939) (see Fig. 3), a glass electrode (timelag 2 to 3 sec.), in close contact with the muscle, was used to examine pH changes consequent upon a 4 sec. tetanus in the frog gastrocnemius.

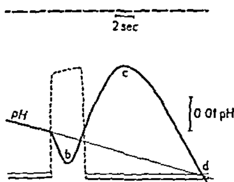


Fig. 3. Changes in pH of gastrocnemius muscle during a tetanus of 3–4 sec. Top record: 2 sec. time marker. The dotted line shows the response of the muscle to stimulation. Phases in the pH change following stimulation are indicated by b, c, and d. Initial pH, 6.9; downward deflection means a fall in pH; the pH calibration is given on the right. Temperature, 20°C. (Dubuisson, 1939).

A series of three pH changes was seen—towards the acid side during shortening; then towards the alkaline side; finally another change to the acid side supervened. The last two changes were mostly postcontraction. Dephosphorylation of ATP sets free acid, while dephosphorylation of creatine phosphate sets free base. The conclusion that the first change is due to ATP breakdown, the second to creatine phosphate breakdown, and the third to lactic acid formation, was supported by the effects of varying the initial pH of the muscle and of poisoning with iodoacetate.

C. The Turnover of ATP during Rest and Contraction; the Use of Radioactive Phosphate

With the introduction of the use of P^{32}, the hope naturally arose that it would now be possible to demonstrate directly *in vivo* increased turn-

over of ATP in the muscle during work, by demonstrating increased labeling of its terminal phosphate group. This hope has so far not been fulfilled, in spite of many attempts in the last 15 years.

Difficulty arises in the assessment in the interior of the fiber of the concentration of the inorganic phosphate with which the ADP must interact; for it is still uncertain how long it takes for P^{32} injected into the bloodstream to come into equilibrium with the extracellular spaces, and how long is needed for equilibration between the outside and the interior of the cell. Estimations of inorganic phosphate in whole muscle of course give both extra-and intracellular; the amounts of inorganic phosphate in the extracellular space is normally only a small proportion of that in the whole muscle, but after injection of labeled phosphate into the blood, the specific activity in the extracellular fluid may be very high for a considerable period, compared to the specific activity inside the cell.

Attempts have been made to calculate (after injection of labeled phosphate into the blood stream of rabbits) what part of the P^{32} of the whole muscle was actually inside the cell, but Kalckar *et al.* (1944) have shown these to be based on unsound assumptions. The latter authors tried the device of washing out the extracellular inorganic phosphate by perfusion of the muscle before measuring the specific activity of the remaining inorganic phosphate. But this procedure too is unsatisfactory. Ennor and Rosenberg (1954) have found that during such perfusion at 0°C., as well as in the control muscle stored in ice, marked changes go on in the specific radioactivity of the ATP, creatine phosphate, and inorganic phosphate as compared with the specific activities of the same compounds in muscle dropped at once into liquid air. Ennor and Rosenberg were however able to show that the speed with which the terminal phosphate of ATP is replaced, even in resting mammalian muscle, is so great that any attempt to find differences as a result of contraction would present great difficulties. On the assessment made by Mommaerts (1950) of the resting activity of mammalian muscle (2×10^{-6} moles per minute per gram muscle), the whole of the ATP content (about 8×10^{-6} moles per gram) would have its terminal phosphate turned over every 4 min. even at rest.

Recently, Fleckenstein *et al.* (1956) have tried to approach the question by experiments *in vitro*, soaking thin frog sartorius muscles in Ringer solution containing radioactive inorganic phosphate. They were able to show a marked increase (ten- to twentyfold) in P^{32} replace-

ment in ATP and in creatine phosphate as a result of raising the temperature from 0° to 20°C.; but application of contracture reagents led to *decrease* in labeling. It seems likely that these happenings depend primarily on permeability phenomena. That there was a very marked barrier to entry of the phosphate was shown by the fact that the specific activity of the inorganic phosphate outside was some 500 times as great as the specific activity of the terminal phosphate of ATP inside, even at the end of the experiment (after 30 min.). One is tempted to think that here too, as in warm-blooded animals, equilibrium was quickly established among the phosphorus compounds inside the cell, and that the effect of raised temperature was to allow increased penetration (with establishment of a new equilibrium) as a result of increased metabolism.

The decrease in labeling during contracture may also be a permeability effect, since much creatine phosphate broke down, and the increased inorganic phosphate in the cell would lead to diminished rate of entry. It may be said that experiments with labeled phosphate give no evidence in favor of rapid breakdown and resynthesis of ATP during contraction, but neither do they provide evidence against it.

D. Attempts to Demonstrate ATP Breakdown *in vivo* as the Primary Reaction

During the last few years, a more direct attack has been made on the problem of ATP participation, in attempts to show its breakdown at a time so soon after stimulation that rephosphorylation of ADP would not yet have taken place. Thus Munch-Petersen (1953), using tortoise muscles fixed by means of liquid propane at —165°C. during the ascending part of the contraction curve, reported a small increase in ADP. Lange (1955) found both ATP and creatine phosphate breakdown, as well as ADP formation, upon contracture by 30 sec. immersion in KCl solution or a few seconds in acetylcholine at 0°C. followed by rapid disintegration in 60% alcohol at 0°C.

It is, however, very striking that a number of cases have been described where measurable tension production took place, without observable ATP or creatine phosphate decrease and with no increase in ADP. Fleckenstein *et al.* (1954b) were the first to draw attention to these phenomena, describing experiments on frog muscle (1.0 to 3.5 sec. tetanus) at 0°C., where ATP, ADP, and creatine phosphate content, measured after chromatographic separation, showed no change.

In later work, these findings were extended (Fleckenstein *et al.* (1954a). An increase in inorganic phosphate was suggested but not conclusively proved. The change to be expected in creatine phosphate in normal muscle with this degree of contraction is only about 3%; but in muscles after treatment with dinitrophenol, in which the creatine phosphate content had already fallen to a low level, and in which a 50% decrease might be expected on contraction, no change was found.

Mommaerts (1955) studied intensively the single slow twitch of turtle muscle at 0°C. (contraction phase 1 to 2 sec.) under anaerobic conditions with maximum tension production. He used an elaborate apparatus for the instantaneous automatic fixation of the muscle (in liquid propane at —180°C.) at any desired point of contraction (Mommaerts and Schilling, 1955). ATP and ADP were estimated by delicate enzymatic methods and no change was found; it was calculated that the method was capable of detecting a 5 to 10% increase and that a 50 to 100% increase in ADP would be expected. Indeed a much greater increase would be expected if the value of Bernhard (1956) for the heat of hydrolysis of ATP under conditions *in vivo* is used. Free creatine also remained unchanged.

The suggestion then arose that other nucleotides, some of which are known to be present in muscle in traces (Bergkvist and Deutsch, 1953), might react directly with the actomyosin, or, after direct reaction of the ATP with actomyosin, might rephosphorylate the ADP. However, the behavior of such nucleotide triphosphates studied *in vitro* with glycerinated fibers gives no grounds for thinking that they are likely to react in any special way with actomyosin *in vivo*—they behave similarly to ATP but less effectively. For rate of hydrolysis with actomyosin and for tension production, Hasselbach (1956) puts them in the order: ATP>CTP>UTP>ITP>GTP[1]; for plasticizing effect, ATP also surpassed any of the others (see Table III). Indeed, Mommaerts gives reasons for believing that by the enzymatic methods of estimation used, such nucleotide triphosphates and the corresponding diphosphates would have been estimated together with the adenine compounds. He suggests that breakdown of ATP may have been concealed by phosphate transfer from an unknown substance—possibly an unknown phosphagen is concerned. He also estimated pyruvate in the control and contracted muscles, with the object of determining whether rephosph-

[1] CTP = cytidine triphosphate, UTP = uridine triphosphate, ITP = inosine triphosphate, and GTP = guanosine triphosphate.

orylation of ADP by reaction with phosphopyruvate might have occurred. No increase in pyruvate was found, but this does not seem entirely conclusive since rapid reduction to lactate of any pyruvate formed might be expected.

Chance and Connelly (1957) have made use of a new and extremely delicate method for ADP estimation. Much previous work had shown that the degree of oxidation of intramitochondrial diphosphopyridine nucleotide changes with addition of phosphate or phosphate acceptors such as ADP. They observed that contraction of a perfused frog sartorius led to increase in the steady-state oxidation of the intramuscular DPN, and gave reasons for believing that ADP is the substance responsible. They have gone on from this to develop a method for the quantitative determination of ADP. The double-beam spectrophotometer was used adjusted to measure the change in absorption at 340 m μ, relative to 386 m μ. The method has the advantage that it uses the intact muscle and no unstimulated paired control is necessary; it is sensitive to 0.001 μ mole per gram muscle and has a response time of 0.1 sec. Calibration curves with known amounts of ADP acting in suspensions of liver mitochondria permitted the assessment that at 7°C., 6 twitches were required to accumulate 0.056 μ moles of ADP, or 0.009 μ moles per twitch, if rephosphorylation was ruled out, as the authors considered likely. This is less than 2% of the expected value.

In all the three cases just described, the authors have expressed the conviction that ADP rephosphorylation during the experiment was excluded or most unlikely. However, the matter is perhaps worth further consideration. It is true that results in the literature make it very probable that in the experiments of Fleckenstein *et al.* and of Chance and Connelly, oxidative rephosphorylation during the contractions did not occur, since for frog muscle at 0°C., D. K. Hill (1940a, b) has shown both oxidative delayed heat (shortest stimulus used, 2 sec. tetanus) and increased O_2 uptake (shortest stimulus used, 5 sec. tetanus) to begin only after relaxation was ended. Perhaps anaerobic rephosphorylation cannot be ruled out with the same likelihood. For anaerobic delayed heat is a small and variable quantity difficult to interpret, since it is the balance of heat production and absorption resulting from simultaneous lactic acid formation and creatine phosphate synthesis. Moreover the degree of delay recorded in lactic acid formation after a short tetanus depends on the nature of the contraction and the conditions; occurrence of post-contraction lactic acid formation as the result of a few twitches

seems not to have been looked for. Investigation of this question under the exact conditions of the present experiments might be helpful. Perry (1956) has pointed out in this connection that the myofibril, site of the ADP production in contraction, is completely surrounded by the sarcoplasm containing the glycolytic system as well as creatine phosphokinase. Very rapid resynthesis here of both ATP and creatine phosphate under the favorable conditions of the first few contractions would not be unexpected. In the experiments of Chance and Connelly neither the glycolytic system nor creatine phosphate is excluded as possibly responsible for rephosphorylation, since only the ADP of the muscle mitochondria is under investigation.

The impasse thus remains that, in spite of all the evidence for the close connection of ATP breakdown with the contraction cycle, in these particular circumstances of low temperature and brief contraction, tension can be produced with no observable ATP participation. It should be remembered that theories regarding ATP dephosphorylation as occurring in relaxation are in just the same difficulty, since Mommaerts for example found no more ADP at the end of relaxation than at any point in rising contraction. This problem awaits solution; meanwhile, the most likely explanation seems to be rephosphorylation of the ADP from some hitherto undetermined source.

V. RELAXATION

Our consideration so far of the contractile machinery has led to the conclusion that high ATP content and low ATPase activity are characteristic of the resting muscle. If this conclusion is accepted, the question arises in an acute form—what keeps the unstimulated muscle in the relaxed condition? It was of course possible to postulate various ways in which stimulation might set off ATPase activity in the previously quiescent filaments, e.g., by affecting permeability and thus overcoming some spatial barrier, by setting free activating Mg^{++} or Ca^{++} ions held in organic combination, or by overcoming the action of some ATPase inhibitor. It is only in very recent years that light has been thrown on this problem by the study of relaxing factors.

Marsh (1951, 1952), in his experiments on muscle homogenates, observed that the removal of the supernatant from the fiber fragments and resuspension of the washed fibers in 0.16 *M* KCl led to a very striking increase in ATPase activity of the fibers and to a condition in which they showed immediate contraction (without any preliminary length-

ening) on addition of ATP. He postulated the presence in the supernatant of a substance which he termed "relaxing factor," protein in nature, and capable of inhibiting actomyosin ATPase; thereby maintaining ATP concentration and favoring the relaxed state. Marsh also observed that after addition of 0.002 *M* $CaCl_2$ to the fresh fiber suspension, only shrinkage, never swelling, was possible; he related this effect to direct Ca^{++} stimulation of the actomyosin ATPase, but we shall see that another explanation is more likely.

Attempts to isolate the Marsh factor quickly followed. Bendall (1954), testing the relaxing effect on glycerinated fiber bundles in presence of 6 m*M* ATP and 4 m*M* Mg^{++} at pH 7, found this effect to follow the myokinase content of his preparations. He prepared pure homogeneous myokinase and found this to be potent in causing relaxation or preventing contraction of the fiber bundles, as well as in inhibiting their ATPase activity.

About the same time, work from A. Szent-Györgyi's laboratory (Goodall and A. G. Szent-Györgyi, 1953; Lorand, 1953) brought evidence that addition to the medium of purified homogeneous creatine phosphokinase, together with creatine phosphate, can relax ATP-contracted glycerinated fibers. In this system, the relaxing effect was only shown at rather acid pH, about 6.2 (obtained by bubbling CO_2); by a change to pH 7, contraction was obtained. Another example of a similar enzyme which can cause relaxation in this fiber system is pyruvate phosphokinase (Moos and Lorand, 1957). In this work, the fiber bundles relaxed upon addition of phosphopyruvate (10 m*M*) in presence of 4 m*M* $MgCl_2$ at pH 7. It was shown that the fibers contained pyruvate phosphokinase.

In all this work, fiber bundles 250–500 μ in diameter were used. Lorand (1953) suggested that relaxation is equivalent to rephosphorylation of actomyosin-bound ADP, and therefore it was to be expected that enzymes producing ATP from ADP would facilitate relaxation.

Perry (1956) and Perry and Grey (1956) have considered the Marsh factor (whether a specific protein or an enzyme system such as those just discussed) as playing its part mainly by binding a metal activator, probably Mg^{++}. (See Section II, D3).

However, it became evident that something more was concerned than providing within the fiber a concentration of ATP supraoptimal for ATPase activity. Kumagai *et al.* (1955) prepared two fractions from muscle extract and showed that both were involved in relaxation.

Fraction A was precipitated by 10 to 20 g. of ammonium sulfate per 100 ml.; the other, fraction B, by 30 to 40 g. per 100 ml. Either alone gave a fairly good relaxing effect in presence of 5 mM ATP and 10 mM Mg^{++}, on relatively fresh single fibers. But with fibers preserved in 50% glycerol for weeks and then exhaustively washed, addition of both factors was necessary. Fraction B contained myokinase and creatine phosphokinase, and fraction A plus myokinase gave relaxation with moderately washed fibers. Nevertheless, something more seemed to be

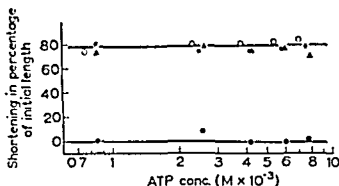
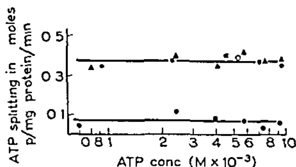


Fig. 4. a. Influence of the creatine phosphate (CP) -CPase system on the dependence of fibrillar contraction on the ATP concentration. Ionic strength about 0.17; pH about 6.3. Oxalate about $4 \times 10^{-3} M$; Mg^{++}, $2\text{–}6 \times 10^{-3} M$. Fibril concentration 4 mg. per millimeter. ▲ with ATP, * with ATP + 0.01 M CP + 3–13 mg. CPase per millimeter, ● with ATP + relaxing factor, and O with ATP + relaxing factor + $4 \times 10^{-3} M$ Ca^{++}.



b. Influence of relaxing factor and the CP-CPase system on the dependence of ATP splitting on ATP concentration. Conditions and symbols as in Fig. 4a except that pH is 7.0 and CPase concentration is 0.03–13 mg. per milliliter (Portzehl, 1957).

contained in fraction B, since A and B together would cause relaxation with old, well-washed fibers for which the combination A plus myokinase was ineffective. They suggested that the fibers used by previous workers had all contained enough of A and B to give relaxation under their conditions.

The situation became clearer with the contributions of Portzehl (1957a) and Briggs and Portzehl (1957). These workers concluded from their observations on the ATPase activity and the degree of shortening of glycerinated myofibrils (not more than 2 μ in diameter) that the ATP-providing enzymatic systems described above cannot be equated with the Marsh factor; for under conditions where such fibrils shortened and hydrolyzed ATP, unaffected by the presence of creatine phosphokinase plus creatine phosphate, addition of Ca-free dialysed muscle extract could abolish both phenomena (see Fig. 4). The relaxing effect of myokinase and of the other enzymatic systems can be explained by the use, in their testing, of fibers still containing traces of Marsh factor, and so thick that in the interior of the bundle the ATP concentration was zero, or at any rate so low that, even in the presence of Marsh factor, it was inadequate for relaxation. In these circumstances, addition of a system which provided for ADP rephosphorylation in the center of the bundle could elicit relaxation.

Thus the essential feature of the action of the Marsh factor would seem to be its inhibition of Mg-activated ATPase. Portzehl (1957b) found that the whole of the factor activity is contained in the particulate fraction of the muscle extract. This would be in agreement with the findings of Kumagai *et al.* (1955), since the cell particles are readily precipitated by ammonium sulfate addition. The Japanese workers indeed had pointed out that their fraction A had a high lipid content and ATPase activity, and might well contain the Kielley-Meyerhof ATPase believed to be associated with the microsomal fraction of the muscle cell. (Kielley and Meyerhof, 1948). Later Ebashi (1958a) showed that granules prepared by centrifuging between 11,000 and 20,000 × g had factor activity; so also had an ATPase preparation made by the Kielley-Meyerhof method. However, he showed that the ATPase activity and the relaxing activity could be independently eliminated by appropriate treatment. Portzehl's results were confirmed by Bendall (1958a) who used washed granules centrifuged down between 18,000 and 80,000 × g; with the centrifugal method of Marsh (1952), he compared their effectiveness with the ineffectiveness of myokinase on the synaeresis of washed myofibrils.

Briggs and Portzehl found that the Marsh factor is effective in abolishing tension (in single fibers only 60 μ in diameter) at pH 6 over a wider range of ATP concentration (above about 0.02×10^{-3} *M*) than at pH 7 (above about 0.2×10^{-3} *M*). This could explain the effect of

pH in the experiments of Lorand (1953) if very little factor were present. It might be mentioned that Moos and Lorand did indeed observe that ADP rephosphorylation could not completely explain their results with phosphopyruvate, since after very extensive washing, no relaxation could be obtained, although the fibers still contained the active phosphokinase. Bendall (1954) also noticed a lack of response to myokinase when old fibers were used.

There is no doubt that with well-washed fresh myofibrils in a medium of very low ionic strength and containing physiological concentrations of ATP (about 5×10^{-3} M), substrate inhibition occurs when the molar ATP concentration exceeds that of Mg^{++} (Perry and Grey, 1956); but the physiological significance of this inhibition is not at present clear. In the experiments of Briggs and Portzehl, Marsh factor could cause drastic fall of tension of single glycerinated fibers at ATP concentrations well below 4×10^{-4} M, with Mg^{++} at 2×10^{-3} M. ATP is certainly necessary for relaxation in the presence of Marsh factor, but as we at present know nothing of the mechanism of factor action, it is impossible to say whether these low yet effective ATP concentrations are causing substrate inhibition as understood in classical enzyme kinetics.

There is general agreement that relaxation in such experiments as those described above is impossible in the presence of traces of Ca^{++} ions. Bozler (1952) found that the relaxation following addition of ATP in high concentration was prevented or abolished by very low Ca concentrations (down to 0.1 mM). He interpreted this effect as due to stimulation of ATPase activity by the Ca^{++}. Bendall (1953a), studying the effects of the Marsh factor, also observed this response to Ca^{++}; he showed that Ca^{++} up to 3 mM did not affect shortening or ATPase activity but that 0.2 mM was enough to prevent relaxation. He concluded that the Ca^{++} effect was directly concerned with the Marsh factor. Moos and Lorand (1957) similarly found low Ca^{++} concentrations to prevent the relaxation induced by addition of phosphopyruvate; while Portzehl (1957a) with glycerinated myofibrils which, as we have seen, responded by lengthening to Marsh factor but not to creatine phosphate plus creatine phosphokinase, found this response to Marsh factor to be abolished by Ca^{++}. Whether there is a connection between these facts and the observation of Perry and Grey (1956) that a trace of Ca^{++} could prevent or greatly diminish the substrate inhibition of Mg-activated ATPase in well-washed fresh myofibrils remains to be clarified.

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marked relaxation was shown, in presence of 4 mM Mg^{++} and ATP. Ca^{++} in low concentration (only about half that of the EDTA) caused reversal of this EDTA relaxation, provided Mg^{++} and ATP were still present; these facts cannot be explained on the simple hypothesis that EDTA acts by forming chelate compounds with the Mg^{++} and Ca^{++} ions. Thus EDTA can cause relaxation in presence of a large excess of Mg^{++} ions, and Ca^{++} ions can overcome this relaxation in presence of some excess EDTA. The suggestion is made by these authors that for the relaxing effect EDTA, Mg^{++}, ATP, and the contractile protein must all enter into a complex formation. Some support is given to this point of view by the difficulty encountered in washing a fiber bundle free from Mg^{++} or EDTA, once it has been in a bath containing these substances. It is also postulated that Ca^{++} combines with the protein but at different sites from those binding Mg^{++}. Bozler has also observed the close imitation by EDTA of Marsh factor effects and has made rather similar suggestions; he has concluded that the relaxed state depends on the formation of an enzymatically inactive ATP-protein complex.

It is significant that Perry and Grey (1956) found low concentrations of EDTA (0.2–0.5 mM) to be very inhibitory to Mg^{++}-activated myofibrillar ATPase, this inhibition being practically independent of the $MgCl_2$ concentration even up to 10 mM. With the Ca^{++}-activated fibrils, inhibition came on as the EDTA concentration approached that of the added Ca^{++}, as one would expect if the Ca^{++} were being removed as the EDTA complex.

VI. CONCLUSION

In the fifty years which have elapsed since Fletcher and Hopkins (1907) opened the modern period of muscle biochemistry, a very complex story has unfolded. The story has taken many unexpected turns and we do not know that we are yet at the end of such surprises. The questions of outstanding interest at the present time are (a) the details of the interaction of ATP and actomyosin in contraction; (b) the mechanism of factor action in relaxation. In both these processes Mg^{++} ions play a part which needs further elucidation; and the dramatic effects of Ca^{++} ions *in vitro* may possibly also have a physiological counterpart.

It may be advantageous to put together here the prerequisites for the various stages we have recognized in the interaction of ATP and actomyosin (see Tables II–IV).

The loosening of linkages, whether evinced in actomyosin solutions

As we have seen, inorganic pyrophosphate can cause relaxation of contracted fiber bundles; even with Mg^{++} present, this relaxation is small (some 20%) unless ATP is also present (Bendall, 1954). Inorganic pyrophosphate inhibits actomyosin ATPase (Bendall, 1954; Perry and Grey, 1956); its main relaxing effect is therefore probably due to increasing ATP concentration when this was the limiting factor—again with fibers not completely freed from Marsh factor. In the high concentrations of pyrophosphate used in the early experiments, the considerable increase in ionic strength of the medium may well have played a part in the change of state of the fiber.

Very little is known about the nature of the factor. It is sensitive to heat and acid (Marsh, 1952) and is also inactivated by treatment with trypsin (but not ribonuclease), desoxycholic acid and some organic solvents (Lorand et al, 1958). Molnar and Lorand (1959) have recently described experiments by Marsh's method in which, with minimal amounts of the microsomal component, myofibrillar relaxation did show potentiation on the addition of a phosphokinase system. They postulate (Lorand *et al.* 1958) that the material responsible for relaxation is a diffusible product of some reaction between the 'microsomes' and the transphorylating system. Gergely (1959) considers that the granule fraction does not account for the whole relaxing activity (tested on single fibers) of the crude muscle extract. Little relaxing activity was found in the granules or in the supernatant alone, but if the supernatant were dialyzed, then with diffusate plus granules original activity was restored. According to Ebashi (1958b) particulate fractions prepared from rabbit liver and heart showed no relaxing effect.

The existence of relaxing factor in or on the microsomal system explains the great difficulty encountered in completely removing the factor from fiber bundles, even the smallest. For a study of the structure of this system in various types of skeletal muscle, the paper of Porter and Palade (1957) may be consulted.

Recently, some very interesting experiments have been done by Watanabe and Sleator (1957) using ethylenediaminetetraacetate (EDTA) as a relaxing agent with glycerinated fiber bundles some 400 μ thick. This work follows on earlier work by Watanabe (1954) and also by Bozler (1954–1955b). For this effect of EDTA, as for relaxation with the natural factor, the presence of both Mg^{++} and ATP is obligatory. Watanabe and Sleator found that, with as little as 0.5 mM EDTA, a

by fall in viscosity or decrease in light scattering, or in actomyosin threads or fibers by increase in extensibility, needs ATP or another ortho-polyphosphate. Engelhardt and his collaborators (Engelhardt, 1946) noticed the effects of ADP and pyrophosphate as well as ATP, and a systematic study by Hasselbach (1956) of resistance to stretch is summarized in Table II. Mg^{++} is very important in this effect (Bozler, 1954–1955a), which is due to the binding of the polyphosphate to the protein, presumably at the Mg^{++}-activated ATPase sites; Ca^{++} plays little part or may be slightly inhibitory.

For ATPase activity, substrate specificity is marked—only nucleotide triphosphates are hydrolyzed, except for inorganic triphosphate, which loses its terminal phosphate at a much lower rate. Both Mg^{++} and Ca^{++} activate actomyosin ATPase, but there is reason to suppose that the sites activated may be different.

TABLE III

COMPARISON OF THE NUCLEOTIDE TRIPHOSPHATES IN THEIR EFFECT ON TENSION PRODUCTION AND IN BEHAVIOR WITH THE MARSH FACTOR[a]

Type	Tension in presence of 10^{-3} M Mg^{++}	Tension in presence of 10^{-2} M Mg^{++}	Minimum M concentration of NTP needed to get relaxation with Marsh factor
ATP	100	100	2.5×10^{-5}
CTP	80	95	7.5×10^{-3}
UTP	—	75[b]	No effect up to 10^{-2}
ITP	30	50	No effect up to 10^{-2}
GTP	15	30	No effect up to 10^{-2}

[a] The tension development with ATP under optimal conditions is put equal to 100. Temperature 21° C., ionic strength 0.1; nucleotide triphosphate (NTP) concentration in columns 2 and 3, 5×10^{-3} M. Single fibers or fiber bundles 160 μ in diameter were used (Hasselbach, 1956).

[b] 5×10^{-3} M Mg.

Only nucleotide triphosphates will evoke contraction; their relative efficiency and their different requirements as regards Mg^{++} concentration are shown in Table III (Hasselbach, 1956). Although Ca^{++} activates the ATPase of the fibers used in measuring tension, little or no contraction is obtained if Mg^{++} is absent (see Fig. 5). It seems likely that only the energy liberated in Mg^{++}-activated ATP breakdown can be used for contraction. In the case of tests on glycerinated fibers, since the latter are in a state corresponding to rigor, it is possible that part of

TABLE II

COMPARISON OF THE PLASTICIZING EFFECT OF POLYPHOSPHATES[a]

Polyphosphate	In the presence of 10^{-5} *M* Mg^{++}	In the presence of 5×10^{-3} *M* Mg^{++}
ATP	3–6	2–3
CTP	50–70	—
UTP	40–50	20
ITP	15	10
GTP	15	2–3
ADP	20–30	10–20
Pyrophosphate	100	10

[a] Resistance to stretch is expressed as percentage of the [illegible]
Temp. 0° C. (Hasselbach, 1956)

the function of the Mg^{++} is to facilitate preliminary plasticization. However, it is interesting to notice that with fresh actomyosin suspensions, Spicer (1951) found Mg^{++} ions to increase the range of ionic strength over which superprecipitation and reversible gel formation took place, while Ca^{++} ions had the opposite effect of restricting this range. Less ATP was needed to cause gel formation when Mg^{++} was present, much more when Ca^{++} was present.

The conditions for relaxation are more exacting than those for con-

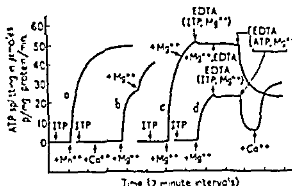


FIG. 5. Effect of ITP in causing shortening under load of glycerinated fiber bundles, in presence of Mn^{++}, Ca^{++}, or Mg^{++}. Concentrations of ITP 8.1 m*M* in a, b, and c; 4.8 m*M* in d. In curve a, Mn^{++} 4 m*M*; in curve b Ca^{++} 4 m*M*, Mg^{++} added 1 mM, then 3 mM. In curves c and d, Mg^{++}, 4 m*M*; EDTA, 4m*M*; and ATP, 4 m*M* (Watanabe and Sleator, 1957).

traction. The presence of the Marsh factor is obligatory, and also Mg^{++} and ATP. In the experiments of Bendall (1953a) with Marsh factor and of Watanabe and Sleator (1957) with EDTA, ATP could not be replaced by ITP (see Fig. 5); in Hasselbach's experiments (1956, see Table III), ATP or CTP was effective, not ITP, UTP, or GTP. Bendall (1958b) found inhibition by EDTA (0.5 m*M*) of nucleotide triphosphatase activity of myofibrils to be much less with ITP than with ATP as substrate. It is interesting to notice that Perry and Grey (1956) observed substrate inhibition to be very slight with ITP. These results seem to underline the importance of the NH_2 group at the 6-position. The requirements in relaxation are (1) cessation of ATPase activity; this is mediated by the Marsh factor; (2) presence of ATP + Mg^{++} to reduce the interaction between myosin and actin. The necessary concentration of ATP seems to be much lower than was previously supposed. The absence of Ca^{++} ions is obligatory.

TABLE IV

EFFECTS OF CALCIUM AND MAGNESIUM IONS AT VARIOUS STAGES IN ATP/ACTOMYOSIN(AM) INTERACTION

Stage	Mg^{++}	Ca^{++}	Reference
Fall in viscosity of AM sols	Accelerates	Slightly inhibits	Baranyi *et al.* (1951)
Fall in light scattering in AM sols	Accelerates	Little effect	Tonomura *et al.* (1953) Blum (1955)
Plasticization of AM threads and glycerinated fibers (increase in extensibility)	Increases effect, especially with some plasticizers	Cannot replace Mg^{++}	Hasselbach (1956)
Gelation and superprecipitation	Extends effective range of ionic strength and of ATP concentration	Restricts these ranges	Spicer (1951)
Tension production	Mg^{++} necessary	Ca^{++} cannot replace Mg^{++}	Bendall (personal communication); see also Portzehl (1954); Watanabe and Sleator (1957)
Shortening of isolated myofibrils	Increases effect	Decreases effect	Bowen (1957)
Relaxation	Necessary	Entirely prevents	Bendall (1953a) Bozler (1954–1955b) Watanabe and Sleator (1957)
ATPase activity at low ionic strength	Activated	Activated	Banga (1941–1942)
Substrate inhibition	Shown with Mg^{++}-activated ATPase	Substrate inhibition with Mg-activated ATPase abolished by addition of Ca^{++}	Perry and Grey (1956)

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In the resting muscle in the living animal, one must picture the Marsh factor as operative. There is abundant ATP and Hasselbach (1957) has recently brought evidence that more than 80% of the muscle magnesium is free (not combined with protein) while about 90% of the calcium is combined, mainly with actin and myosin, but partly in the cell particles. Even the remainder may be combined with soluble proteins. The initial effect of the stimulus is still obscure; but presumably it results in partial or complete inactivation of the factor. ATPase activity can then begin and during the balance between enzymatic depletion of ATP and the arrival by diffusion of fresh ATP, the actin-myosin links are continually made and broken. When the stimulus ceases, the factor again comes into play, the ATP concentration rises, and the initial state of relative dissociation of the two proteins is restored. How exactly the Marsh factor comes in and out of play is one of the most obscure problems at the present time.

The physical machinery operated by the nucleotide triphosphate and its enzyme sites seems thus to be more discontinuous in contraction than in relaxation. For during shortening, the cross linkages between the proteins form and reform like a ladder, the rungs of which themselves climb; while during lengthening, the protein chains are free to yield to the elastic recoil of noncontractile components and to slide continuously upon one another back to the position of rest.

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CHAPTER III

Biochemistry of Sarcosomes[1,2]

E. C. Slater

I. INTRODUCTION

It has been known for more than one hundred years, since the publication of the book "Allgemeine Anatomie" by Henle (1841), that the

[1] The following abbreviations are used: DPN^+, DPNH, oxidized and reduced diphosphopyridine nucleotide; AMP, ADP, ATP, adenosine mono-, di-, and triphosphate; GDP, GTP, guanosine di-, and triphosphate; P_i, inorganic orthophosphate; PP, inorganic pyrophosphate; GP, α-glycerol phosphate; DHAP, dihydroxyacetone phosphate; Co A or A.SH, coenzyme A; $\overline{S-S}$, α-lipoic acid (6-thioctic acid); fp_I, flavoprotein (diaphorase); fp_{II}, flavoprotein (succinic dehydrogenase); cyt., cytochrome; $\sim$P, site of phosphorylative step in respiratory chain; α-Kg, α-ketoglutarate; glut., glutamate; asp., aspartate; OxAc, oxaloacetate; succ., succinate; mal., malate; DNP, 2,4-dinitrophenol; EDTA, ethylenediaminetetraacetate; TRIS, tris(hydroxymethyl)aminomethane or 2-amino-2-hydroxymethyl-propane -1, 3-diol.

[2] In this chapter, the word "sarcosome," introduced by Retzius (1890) is used synomously with "muscle mitochondria." The author does not accept the attempt by Kitiakara and Harman ([illegible] somes, the latter being descril[illegible] soluble in organic solvents. N[illegible] of the word sarcosome to incl[illegible] fat particles, as suggested by Chappell and Perry (1953). If this proposal were adopted it would mean including the granules of the sarcoplasmic reticulum, which appear to give rise to microsomes in other tissues, under the name sarcosome, and could cause considerable confusion.

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muscle activity over short periods. The muscles required for sustained activity are predominantly aerobic in their metabolism. Keilin (1925) showed that among all the large number of tissues examined, the thoracic muscles of insects were the ones with the highest concentrations of cytochrome.

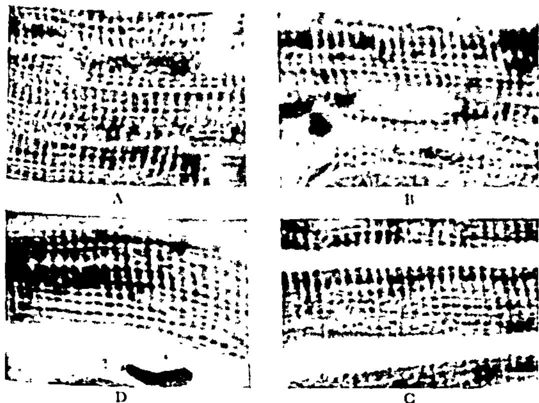


FIG. 1. Longitudinal sections of rat heart muscle. Tissue fixed with Helly's fluid (24 hr.), then treated with 2.5% $K_2Cr_2O_7$ for 24 hr. at 37°C. Paraffin sections, 5 μ. Stained with Altmann's aniline - acid fuchsin.

The large bodies are erythrocytes. All photomicrographs show rod-shaped intermyofibrillar sarcosomes. A and B show spherical perinuclear sarcosomes. In the right lower corner of A is an intercalated disk, free of sarcosomes. In D, some of the sarcosomes are a little displaced from their normal positions. A direct demonstration that the transverse rows of sarcosomes lie at the level of the anisotropic striations was possible by polarization microscopy of favorable areas. This was confirmed by the observation of Z-bands between the sarcosomes. [Reproduced from Cleland and Slater (1953a) by kind permission of the Company of Biologists.]

Regaud (1909), Holmgren (1910), and Bullard (1913, 1916) showed that in heart muscle, the granules were very regularly arranged between the myofibrils, with one granule opposite each anisotropic disk. They also described the presence of a few granules at the pole of the nucleus. This distribution was recently confirmed for rat heart by

sarcoplasm of striated muscle contained granules. The unusually large and abundant granules of insect muscle were first observed by Aubert (1853).

Kölliker (1857, 1888) made a thorough study of these granules, which he called "interstitial granules," and showed that they were widely distributed in the animal kingdom. In the second of these papers, he described the separation of the granules from insect muscle and studied the effect of various treatments, such as suspension in a hypotonic medium, on the appearance of the granules under the microscope. This paper is of considerable historical interest, since it foreshadowed by sixty or seventy years much of the recent work on the structure of these and related granules.

Kolliker's studies were followed by further detailed investigations by a number of distinguished cytologists. Retzius (1890) extended Kölliker's descriptions and introduced the name "sarcosome" in the following words:

"They exist in many animals in different positions and arrangements —frequently in longitudinal rows, as has been shown by Kölliker—and they constitute, as the latter has already pointed out, 'Körperchen sui generis'; they are not fat granules, as several authors have maintained, even recently. Because of their pronounced and characteristic properties (resistance to reagents, specific staining properties, etc.) and also in order to distinguish them clearly from pathologically formed fat granules . . . I have named the normal interstitial, or more correctly intercolumnar, Körperchen, as 'sarcosomes'."

Holmgren (1910) and Bullard (1913, 1916) made detailed studies of the granules present in heart muscle. Holmgren classified striated muscles as falling in two types, depending upon the distribution of the granules, and recognized that these two classes correspond to two physiological types. Muscles with granules at the level of the isotropic bands of the myofibrils are those with intermittent activity, such as the skeletal muscle of many vertebrates and invertebrates. Granules at the level of the anisotropic bands are found in muscles required for a sustained activity, such as the flight muscles of birds and insects, and the heart muscle of vertebrates. These types of muscle contain more and larger granules than the first type.

In more recent years, it has become apparent that Holmgren's classification has a biochemical basis. The muscles with granules at the level of the isotropic bands are rich in glycolytic enzymes, sufficient for

came from work on another tissue, liver. Bensley and Hoerr (1934) and Claude (1944) introduced differential centrifugation as a method of separating the various structural components in the cell, and Hogeboom *et al.* (1946) showed that the mitochondria contained the respiratory enzymes. This work was soon followed by the recognition that the mitochondria contained all the enzymes necessary for the complete oxidation of pyruvate to carbon dioxide and water and also for coupling this oxidation with the esterification of inorganic phosphate.

Although as early as 1909 Regaud had concluded on the basis of staining reactions that the sarcosomes were identical with mitochondria, the identification of the latter as the respiratory granules in liver did not allow the conclusion, without further examination, that the sarcosomes were the respiratory granules of muscle. The myofibrils constitute more than half the weight of the muscle cell. In view of the fact that the myofibril is intimately concerned with the utilization of ATP in the functioning muscle, the possibility could not be excluded that it also contained the enzymatic mechanism for the synthesis of ATP by oxidative phosphorylation. It was necessary to identify the respiratory component of the muscle by direct experiment.

The methods employed were essentially those used by earlier workers with liver, namely the separation of the disintegrated muscle by differential centrifugation into microscopically identifiable fractions and measurements of their respiratory activity. The first muscle studied in this way was the thoracic muscle of the blowfly. Watanabe and Williams (1951) isolated the sarcosomes and showed that the specific activity of cytochrome oxidase was twice that of the whole muscle.

Slater (1950) isolated granules by differential centrifugation from extracts of cat heart and showed that they brought about oxidative phosphorylation. However, the granules were not identified microscopically. Harman (1950) examined microscopically heart-muscle "cyclophorase" (Green, 1951) preparations and concluded that mitochondria were the only pre-existent structural unit recognizable. However, since the major proportion by weight of a "cyclophorase" preparation consists of material derived from myofibrils, it was necessary to separate the myofibrils from the granules before the question as to which component of the muscle contained the respiratory enzymes could be settled. This separation was done by Harman and Feigelson (1952) who showed that the separated mitochondria possessed respiratory activity. Cleland and Slater (1953b) separated cat heart muscle

Cleland and Slater (1953a); a photomicrograph from this paper is reproduced in Fig. 1[3].

After 1916, comparatively little attention was paid to the sarcosomes. In studies of the structure of muscle, emphasis was placed more on the detailed structure of the myofibril, and this emphasis continued after the advent of the electron microscope until very recently. Many of the studies with the electron microscope were, in fact, done with those types of muscle which contain few granules.

II. FUNCTION OF SARCOSOMES

A. Identification of Sarcosomes as Respiratory Granules of Muscle

During the 1920's and 1930's, the elucidation of the main energy-yielding reactions of the cell, particularly in muscle, occupied the attention of a number of the leading biochemists of the day. Rabbit skeletal muscle, containing relatively few granules, but rich in soluble glycolytic enzymes in the sarcoplasm, was used in many studies of the glycolytic pathway, while actively respiring muscle, such as pigeon breast or heart muscle, was used for the study of respiratory enzymes. Muscle minces were often used in studies of intermediary metabolism but for the more detailed examination of the enzyme systems involved, a suspension of very small particles isolated from heart muscle was found more convenient. This type of preparation had been introduced by Battelli and Stern as long ago as 1912 for the study of succinic oxidase and indophenol oxidase. These workers recognized that the main respiration of the cell took place on these particles. A similar heart-muscle preparation has been used by Keilin and his school since about 1925 for the study of the cytochrome system. Although it was recognized that these particles contained a delicately organized complex enzyme system, which must be derived from some pre-existing structure in the intact muscle, no attempt was made to determine its origin in the muscle.

The great impetus to this aspect of the study of respiratory enzymes

[3] This very regular arrangement of the intermyofibrillar granules, one opposite each anisotropic disk, is not shown in recently published electron micrographs of heart muscle (e g. Kisch, 1956). The author does not feel competent to discuss whether this discrepancy is due to the greater resolution of electron microscopy or to displacement of the granules from their normal positions during preparation of the material for electron microscopy.

Studies by Chappel and Perry (1953) of the distribution of succinic oxidase and pyruvic oxidase and by Harman and Osborne (1953) of α-ketoglutaric oxidase in pigeon breast muscle gave similar results.

The respiratory activities of different types of muscles were compared with the mitochondrial density by Paul and Sperling (1952). The results with α-ketoglutarate as substrate, assembled in Table II, show that white muscle containing very few mitochondria has a low respiratory activity, while red muscle which is rich in mitochondria has a high respiratory activity. The red color is due to myoglobin, which assists the transfer of oxygen from the blood to the respiratory enzymes in the mitochondria. In the rabbit, the highest respiratory activity is found in the heart and the diaphragm, while the skeletal muscle has a very low activity. This is the explanation of the lack of "staying power" of a rabbit. Short periods of muscular activity are maintained by the high concentration of glycolytic enzymes in rabbit muscle, which is widely used by the biochemist for preparing these enzymes. The low respiratory activity of the breast muscle of the non-flying chicken in comparison with that in the pigeon is also noteworthy.

Lawrie (1952) has reported in a more quantitative form the correlation between the myoglobin content and respiratory activity[4] (Table III). In general, high myoglobin content is associated with high respiratory activity. Lawrie suggests that the relatively low myoglobin content of horse heart and of pigeon breast muscle, despite high enzyme activity, may be explained by their possession of an excellent supply of oxygen from the blood. This supply is continually replenished by the characteristic repetitive contractions, thus partly dispensing with the need for an oxygen store. In contrast, the slow wing movements of the Manx shearwater, as compared with the more rapid ones of the pigeon, are reflected in the higher concentration of myoglobin in its pectoral muscle. The enzymatic activity of the psoas muscle of the whale, which is very low compared with the myoglobin content, helps to explain the diving capabilities of this mammal, for a low rate of oxygen utilization increases the length of time during which its relatively meager store of myoglobin-bound oxygen could be effective.

Lawrie (1953) has shown that the myoglobin content of the leg

[4] It should be pointed out that, in contrast to the values in Table II, the respiratory activities in Table III do not relate to the whole muscle, but to the isolated sarcosomal fragments. Thus, no account is taken here of differences in the concentration of sarcosomes in the different muscles.

into various fractions, which were identified microscopically, and determined the distribution of the enzyme activity. The results, which are summarized in Table I, show that the isolated sarcosome fraction

TABLE I
DISTRIBUTION OF PROTEIN AND RESPIRATORY ENZYME ACTIVITY IN FRACTIONS ISOLATED FROM CAT HEART MUSCLE[a]

	Protein		α-ketoglutaric oxidase		Cytochrome c oxidase	
	(mg.)	(%)	(Q_{O_2})	(%)	(Q_{O_2})	(%)
Homogenate	—	100	12	100	220	100
Myofibril fraction[b]	420	62	7	36	85	24
Sarcosomes	62	9	70	55	1590	66
Smaller granules[c]	33	5	19	8	580	13
Soluble	164	24	—	—	49	6

[a] Data from Cleland and Slater, 1953b.
[b] Contains also nuclei, erythrocytes, and some sarcosomes.
[c] Contains sarcosomes.

has 6–7 times the specific respiratory activity of the whole muscle. The activities also found in the myofibril fraction and in unidentified smaller granules are very likely due to contaminating sarcosomes.

TABLE II
RESPIRATORY ACTIVITY OF DIFFERENT TYPES OF MUSCLE, CORRELATED WITH MITOCHONDRIAL DENSITY AND WITH THE COLOR OF THE MUSCLE[a]

Animal	Muscle	Color of muscle	Mitochondrial density	Respiratory[b] activity (μl. O_2/hr./g. wet wt. tissue)
Rabbit	Back	White	0	35
Chicken	Breast	White	0	110
Rabbit	Gastrocnemius	White	0	110
Rabbit	Soleus	Reddish	+	85
Rat	Leg	Reddish	+	85
Bat	Forearm	Red	++	185
Rabbit	Diaphragm	Red	++	545
Mallard	Breast	Red	+++	875
Rabbit	Heart	Red	++++	1110
Pigeon	Breast	Red	++++	1830

[a] Calculated from Paul and Sperling, 1952.
[b] α-Ketoglutarate as substrate.

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[4] It should be pointed out that, in contrast to the values in Table II, the respiratory activities in Table III do not relate to the whole muscle, but to the isolated sarcosomal fragments. Thus, no account is taken here of differences in the concentration of sarcosomes in the different muscles.

TABLE III

CYTOCHROME OXIDASE ACTIVITY OF PREPARATIONS ISOLATED FROM VARIOUS TYPES OF MUSCLES, CORRELATED WITH MYOGLOBIN CONTENT OF THE MUSCLE[a]

Animal	Muscle	Myoglobin content (% wet wt.)	Cytochrome c oxidase activity ($\mu l. O_2$/mg. fat-free dry wt/hr.)
Rabbit	Psoas	0.02	250
Hare	Psoas	0.16	650
Sheep	Psoas	0.35	950
Pig	Psoas	0.43	1000
Elephant	Psoas	0.46	800
Ox	Psoas	0.60	1200
Blue whale	Psoas	0.84	600
Horse	Psoas	0.71	1700
Horse	Diaphragm	0.61	1700
Horse	L. dorsi	0.47	900
Horse	Heart (5 months before birth)	0.02	260
Horse	Heart (6 weeks before birth)	0.10	1500
Horse	Heart (12 months after birth)	0.27	2700
Horse	Heart (2–12 years after birth)	0.33	2800
Manx Shearwater	Pectoral	0.69	1800
Pigeon	Pectoral	0.22	2300

[a] Data from Lawrie, 1952.

muscles of the rat and fowl is increased by continuous exercise. There was no change in the pectoral muscle.

The various studies reported above have firmly established that the granules which have been described by the cytologist under the names "interstitial granules," "sarcosomes," or "mitochondria" are the respiratory granules of muscle corresponding to mitochondria in other tissues. Tables II and III show why mammalian heart muscle and pigeon breast muscle have been the materials most used by biochemists in studying respiratory enzymes.

B. FUNCTION OF SARCOSOMES

The sarcosomes lie very close to the myofibrils, perhaps in direct contact. They are, therefore, very well situated to supply ATP to the myofibril, and this is their main function in the muscle cell. ATP is synthesized by a process known as oxidative phosphorylation.

The substances which are extracted from the blood and oxidized by the normal human heart have been determined by Bing *et al.* (1954; Bing, 1954). The results for the postabsorptive state, given in Table IV,

TABLE IV
SUBSTRATES OF HUMAN HEART[a]

Substance	Per cent of myocardial O_2 usage
Glucose	18.0
Pyruvate	0.5
Lactate	16.5
Fatty acids	67.2
Amino acids	5.6
Ketones	4.3
	112.1

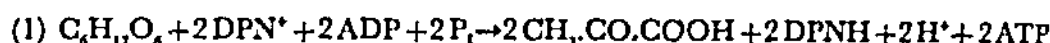
[a] Data from Bing *et al.*, 1954.

show that more than a half of the oxygen consumption is due to the oxidation of fatty acids. Most of the remaining oxygen uptake is accounted for by oxidation of glucose and lactate; amino acids are unimportant as oxidizable substrate. The great versatility of the myocardium in the use of its fuel supply may be regarded as an important safety factor.

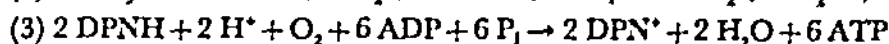
The figures in Table IV are based on the assumption that the substrates are completely oxidized. The calculated total (112.1%) shows that only a small proportion (equivalent to 10.8% of the O_2 usage) of the total is otherwise metabolized. The utilization of fat is greatly increased after a high fat intake, without a corresponding increase of O_2 usage, showing that the fatty acids can be stored. This enables the heart to protect its energy production against a sudden decline in food supply. The utilization of amino acids increases sharply (up to 40% of the total) if the blood amino acid level is increased somewhat by infusion.

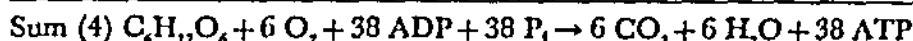
In the fly, glycogen appears to provide the major vehicle for storage of flight energy (Wigglesworth, 1949). In other insects, e.g. the locust, fat deposits form the main reserves (see Sacktor, 1955, for review). In most muscles, the complete oxidation of glucose to carbon dioxide and water takes place in three stages[5].

[5] The molecule of water formed by reaction of a molecule of ADP with one of H_3PO_4 has been ignored in these and subsequent equations.


(1) $C_6H_{12}O_6 + 2\,DPN^+ + 2\,ADP + 2\,P_i \rightarrow 2\,CH_3.CO.COOH + 2\,DPNH + 2\,H^+ + 2\,ATP$

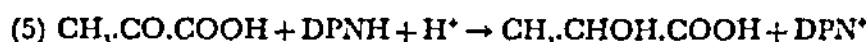

(2) $2\,CH_3.CO.COOH + 5\,O_2 + 30\,ADP + 30\,P_i \rightarrow 6\,CO_2 + 4\,H_2O + 30\,ATP$


(3) $2\,DPNH + 2\,H^+ + O_2 + 6\,ADP + 6\,P_i \rightarrow 2\,DPN^+ + 2\,H_2O + 6\,ATP$


Sum (4) $C_6H_{12}O_6 + 6\,O_2 + 38\,ADP + 38\,P_i \rightarrow 6\,CO_2 + 6\,H_2O + 38\,ATP$

According to our present knowledge, it appears that reaction (1) (glycolysis) takes place in the sarcoplasm, while reactions (2) and (3) take place in the sarcosomes. Thus 36/38 (95%) of the total ATP formed during the oxidation of glucose is made in the sarcosomes. These sarcosomal reactions are described in greater detail below.

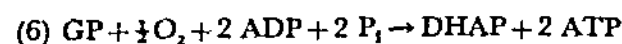
In the absence of oxygen, or in muscles relatively deficient in sarcosomes, the DPNH and pyruvic acid formed in reaction (1) react together to form lactic acid


(5) $CH_3.CO.COOH + DPNH + H^+ \rightarrow CH_3.CHOH.COOH + DPN^+$

As shown in Table IV, human heart muscle utilizes lactate to about the same extent as glucose. Bogue *et al.* (1935) showed that the normal dog heart, in a heart-oxygenator circuit, uses about three times as much lactate as glucose. The first reaction in the oxidation of lactate is probably the reverse of reaction (5), which is catalyzed by lactic dehydrogenase present in the sarcoplasm. The pyruvic acid produced then enters reaction (2).

The importance of reaction (1) for skeletal muscle is shown by the fact that contraction stops owing to failure of ATP supply if this reaction is poisoned by iodoacetic acid. However, the heart will contract for an indefinite period after poisoning with iodoacetic acid, provided that oxygen is available (Evans, 1936). Poisoning with iodoacetic acid stops the utilization of glucose, but the utilization of the other substrates listed in Table IV proceeds unimpaired.

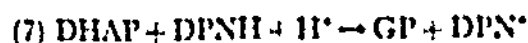
Sarcosomes can also oxidize α-glycerol phosphate to dihydroxyacetone phosphate (Green, 1936; Tung *et al.*, 1952; Sacktor and Cochran, 1956, 1957a) by a reaction[4] which does not involve DPN.


(6) $GP + \frac{1}{2}O_2 + 2\,ADP + 2\,P_i \rightarrow DHAP + 2\,ATP$

The DHAP formed can be reduced to GP by DPNH by the sarco-

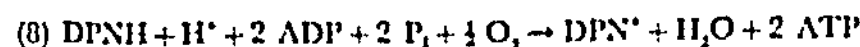
[4] Since Sacktor and Cochran (1956) have found a P:O ratio of 1.4 with α-glycerol phosphate, compared with 1.5 with succinate, for sarcosomes isolated from the housefly *Musca domestica*, it is assumed that there are two phosphorylative steps in the oxidation of α-glycerol phosphate.

plasmic α-glycerol phosphate dehydrogenase (Chefurka, 1954, 1958; Sacktor and Cochran, 1957b).


(7) $DHAP + DPNH + H^+ \rightarrow GP + DPN^+$

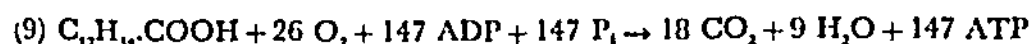
Chefurka (1958) has shown that in the flight muscle of the housefly, reaction (7) is much more rapid than reaction (5), which explains why lactate is not formed by this muscle.

It is interesting to note that the sum of reactions (6) and (7) describe an oxidative phosphorylation of DPNH [reaction (8)].


(8) $DPNH + H^+ + 2\ ADP + 2\ P_i + \frac{1}{2}\ O_2 \rightarrow DPN^+ + H_2O + 2\ ATP$

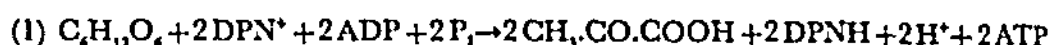
Reaction (8) would proceed in the presence of sarcosomes, sarcoplasm, and a catalytic amount of GP or DHAP. It is made possible by the fact that reaction (7) but not reaction (6) involves DPN.

The sarcosomes are also able to bring about the oxidation of fatty acids. The complete oxidation of stearic acid, liberated from neutral fat by hydrolysis, is accompanied by the formation of a large amount of ATP [equation (9)].


(9) $C_{17}H_{35}.COOH + 26\ O_2 + 147\ ADP + 147\ P_i \rightarrow 18\ CO_2 + 9\ H_2O + 147\ ATP$

It should be understood that the values given for the number of molecules of ATP synthesized in the various reactions have been calculated on the assumption that there occur no side reactions, leading to loss of ATP. As is discussed below, these side reactions are found with isolated sarcosomes, but it is not yet clear to what extent they take place *in vivo*. It should also be understood that not all of the ATP produced by the sarcosomes will be available for the production of mechanical energy by the myofibril. Some energy is needed for the maintenance of the difference in ionic composition inside and outside the cell and for the synthesis of components of the cell, such as proteins.

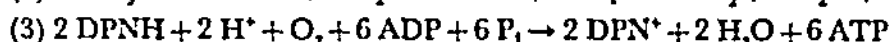
Equations (4) and (9), which describe the complete oxidation of glucose and a typical fatty acid, respectively, include ADP and P_i as components of the reactions. In other words, the phosphorylation of ADP to ATP by inorganic phosphate is obligatorily coupled with respiration and the latter will cease when the supply of ADP or inorganic phosphate is exhausted. Thus, the hydrolysis of ATP, which occurs in the energy-utilizing reactions of the cell, is necessary for the continuous operation of respiration. This phenomenon, known as respiratory control, was first clearly demonstrated by Lardy and Wellman (1952) with



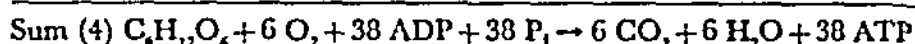
$$(1)\ C_6H_{12}O_6 + 2\,DPN^+ + 2\,ADP + 2\,P_i \rightarrow 2\,CH_3.CO.COOH + 2\,DPNH + 2\,H^+ + 2\,ATP$$



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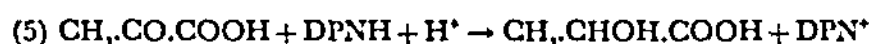
$$(3)\ 2\,DPNH + 2\,H^+ + O_2 + 6\,ADP + 6\,P_i \rightarrow 2\,DPN^+ + 2\,H_2O + 6\,ATP$$



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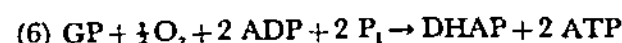


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$$(6)\ GP + \tfrac{1}{2}O_2 + 2\,ADP + 2\,P_i \rightarrow DHAP + 2\,ATP$$

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Respiratory control in the heart muscle, *in vivo*, has been demonstrated by Melrose *et al.* (1955), during studies of surgical techniques for the heart. While the vital centers were protected by perfusion with a heart-lung machine or by lowering the body temperature, blood was excluded from the heart, and cardiac arrest was induced by injection of potassium citrate into the coronary circulation. Within 5 seconds after the injection, the heart action was arrested; it became quite flaccid, and could be freely handled. While the heart was stopped without coronary blood flow for 15 minutes, it remained pink, and determinations of the oxygen content of the blood obtained from the coronary sinus showed that very little oxygen was utilized. The heart action could be restored after 15 minutes by allowing the blood to re-enter the heart.

This seems to show quite clearly that the arrest of heart action, caused by injection of potassium citrate, stops the intracellular respiration, presumably because ATP is no longer broken down to ADP (cf. Clark *et al.*, 1931). Under these conditions, the structure of the heart is quite stable, and it can be restored to normal activity. If, however, the heart was stopped by asphyxia, induced by stopping the coronary flow, without the addition of potassium citrate, the recovery was very slow and only partial after 5 minutes. In this case, the heart beat stops because all the ATP is broken down to ADP, which cannot be rephosphorylated because of the absence of oxygen. In the absence of ATP, the heart soon suffers irreversible damage.

III. DETAILED MECHANISMS OF ENERGY-YIELDING REACTIONS IN SARCOSOMES

No attempt has been made in this section to document by references to the literature statements which are found in many textbooks of biochemistry.

A. Oxidation of Pyruvic Acid

The main features of the mechanism of the oxidation of pyruvic acid, summarized in reaction (2), are now well-established. It is convenient to consider reaction (2) in three parts: (1) the oxidation of the carbon atoms to CO_2 (carbon pathway); (2) the oxidation of hydrogen atoms to water (hydrogen or electron pathway); and (3) the reaction of ADP with inorganic phosphate to form ATP (oxidative phosphorylation).

liver mitochondria, and has been recently emphasized by Chance and Williams (1956). Although isolated heart muscle sarcosomes usually show appreciable respiration in the absence of ADP (possibly because they are damaged during isolation), a very considerable stimulation of the respiration by added phosphate acceptor can be demonstrated (Fig. 2).

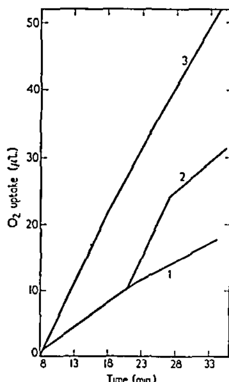


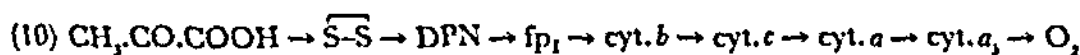
FIG. 2. Respiratory control in guinea pig heart sarcosomes, isolated in 0.23 *M* sucrose, 0.005 *M* ATP, pH 7.4, and suspended in 0.25 *M* sucrose, 0. 001 *M* ATP, pH 7.4. Reaction mixture: phosphate, pH 7.4, 0. 016 *M*; NaF, 0.012 *M*; $MgCl_2$, 0.006 *M*; KCl, 0.083 *M*; ATP, 10^{-4} *M*; EDTA, 0. 001 *M*; glutamate (DL), 0.007 *M*; sarcosomes, 0.6 mg. protein/ml. Reaction vol., 1 ml.; temp., 25°C. Curve 1, no urther addition. Curve 2, ADP (2.7 μmoles) added at 19 minutes. Curve 3, 0.02 *M* glucose +hexokinase present from beginning. The measurement of O_2 uptake com- [illegible] mann.)

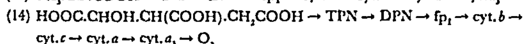
The magnitude of the respiratory control which is achieved, *in vivo*, is well illustrated by some results obtained by Asmussen *et al.* (1939) on the relationship between the work performed and the oxygen consumed by the working leg muscles of a man. The rate of O_2 consumption was increased by a factor of 65 during heavy work.

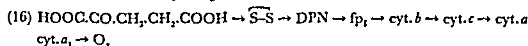
number of reactions stepwise, with the net result that energy is liberated in twelve separate portions. Thus, 12 molecules of ATP are liberated in reactions (11)–(19).

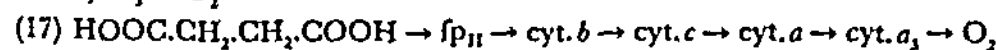
2. *Hydrogen or Electron Pathway*

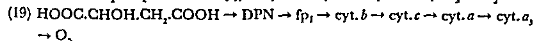
In the oxidation reactions of the Krebs cycle, the hydrogen atoms (or the electrons derived from them) do not react directly with oxygen, but pass through a series of hydrogen or electron carriers (the respiratory chain). The number of carriers and the mechanism of their action are not known so well as the carbon pathway, but the main features are clear. In the following schemes, the arrows denote the pathway of the hydrogen atoms or electrons between substrate and oxygen. The number given to each scheme corresponds to the number of the chemical equations above.


(10) $CH_3.CO.COOH \rightarrow \overparen{S{-}S} \rightarrow DPN \rightarrow fp_I \rightarrow cyt.b \rightarrow cyt.c \rightarrow cyt.a \rightarrow cyt.a_3 \rightarrow O_2$

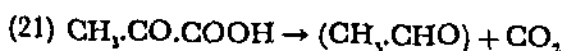

(14) $HOOC.CHOH.CH(COOH).CH_2COOH \rightarrow TPN \rightarrow DPN \rightarrow fp_I \rightarrow cyt.b \rightarrow$ $cyt.c \rightarrow cyt.a \rightarrow cyt.a_3 \rightarrow O_2$

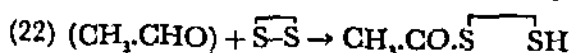

(16) $HOOC.CO.CH_2.CH_2.COOH \rightarrow \overparen{S{-}S} \rightarrow DPN \rightarrow fp_I \rightarrow cyt.b \rightarrow cyt.c \rightarrow cyt.a$ $cyt.a_3 \rightarrow O_2$

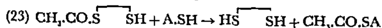

(17) $HOOC.CH_2.CH_2.COOH \rightarrow fp_{II} \rightarrow cyt.b \rightarrow cyt.c \rightarrow cyt.a \rightarrow cyt.a_3 \rightarrow O_2$


(19) $HOOC.CHOH.CH_2.COOH \rightarrow DPN \rightarrow fp_I \rightarrow cyt.b \rightarrow cyt.c \rightarrow cyt.a \rightarrow cyt.a_3$ $\rightarrow O_2$

The first hydrogen-transfer step of reaction (10) is presented here very schematically. In fact, the carbon pathway and the hydrogen pathway are in this first step so closely linked that their separation, as has been done here, is scarcely justified. (The same comment might have to be made concerning the other first steps, when the mechanisms become clearer.) The first step of reaction (10), written in full, is shown in equations (21)–(23).


(21) $CH_3.CO.COOH \rightarrow (CH_3.CHO) + CO_2$

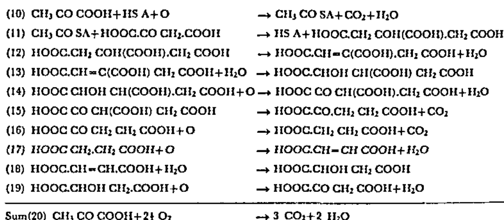

(22) $(CH_3.CHO) + \overparen{S{-}S} \rightarrow CH_3.CO.\overparen{S\quad SH}$


(23) $CH_3.CO.\overparen{S\quad SH} + A.SH \rightarrow \overparen{HS\quad SH} + CH_3.CO.SA$

Diphosphothiamine is involved in equation (21). The product written $(CH_3.CHO)$ is some unknown bound form of acetaldehyde which is

1. *Carbon Pathway*

The series of reactions leading to the formation of three molecules of CO_2 from the three carbon atoms of pyruvic acid are described by reactions (10)–(19).



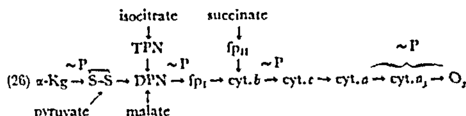
(10)	$CH_3\,CO\,COOH + HS\,A + O$	$\rightarrow CH_3\,CO\,SA + CO_2 + H_2O$
(11)	$CH_3\,CO\,SA + HOOC.CO\,CH_2.COOH$	$\rightarrow HS\,A + HOOC.CH_2\,COH(COOH).CH_2\,COOH$
(12)	$HOOC.CH_2\,COH(COOH).CH_2\,COOH$	$\rightarrow HOOC.CH{=}C(COOH).CH_2\,COOH + H_2O$
(13)	$HOOC.CH{=}C(COOH)\,CH_2\,COOH + H_2O$	$\rightarrow HOOC.CHOH\,CH(COOH)\,CH_2\,COOH$
(14)	$HOOC\,CHOH\,CH(COOH).CH_2\,COOH + O$	$\rightarrow HOOC\,CO\,CH(COOH).CH_2\,COOH + H_2O$
(15)	$HOOC\,CO\,CH(COOH)\,CH_2\,COOH$	$\rightarrow HOOC.CO.CH_2\,CH_2\,COOH + CO_2$
(16)	$HOOC\,CO\,CH_2\,CH_2\,COOH + O$	$\rightarrow HOOC.CH_2\,CH_2\,COOH + CO_2$
(17)	$HOOC\,CH_2.CH_2\,COOH + O$	$\rightarrow HOOC.CH{-}CH\,COOH + H_2O$
(18)	$HOOC.CH{=}CH.COOH + H_2O$	$\rightarrow HOOC.CHOH\,CH_2\,COOH$
(19)	$HOOC.CHOH\,CH_2.COOH + O$	$\rightarrow HOOC.CO\,CH_2\,COOH + H_2O$
Sum(20)	$CH_3\,CO\,COOH + 2\tfrac{1}{2}\,O_2$	$\rightarrow 3\,CO_2 + 2\,H_2O$

The first molecule of CO_2 comes free in the first reaction, the oxidative decarboxylation of pyruvic acid to acetic acid. However, the acetic acid is not formed as free acetic acid, but is bound to the thiol group of coenzyme A. The acetyl group is then added to a 4-carbon compound (oxaloacetic acid) in reaction (11) to form a 6-carbon compound (citric acid). Reactions (12)–(16) form a device for the oxidation of the two carbon atoms introduced with the acetyl group to CO_2. (Actually, the carbon atoms which are introduced are not the ones which are oxidized.) Reactions (17)–(19) regenerate the oxaloacetic acid which can re-enter reaction (11).

Thus reactions (11)–(19) describe a cyclic process (the Krebs cycle) in which acetyl-CoA enters the cycle and CO_2 and H_2O come out. All the compounds containing 6, 5, or 4 carbon atoms in equations (11)–(19) remain in the cycle, since each molecule consumed is regenerated.

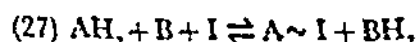
At first sight, the Krebs cycle may appear to be a very cumbersome device to oxidize the two carbon atoms of the acetyl group to carbon dioxide. However, when it is realized that the production of carbon dioxide is not the function but the incidental result of the cycle, the neatness and beauty of the mechanism becomes clear. The function of the oxidation of acetic acid in the cell is not in the first place to remove an unwanted product, but to provide energy in an usable form. The chemical possibilities of oxidizing free acetic acid are rather limited. But by adding it to a larger compound, it is possible to carry out a

14 molecules of ATP. None appear to be formed in the first step of each chain. While the exact location of the phosphorylative steps in the respiratory chain is not known with certainty, there is increasing evidence in favor of something like the scheme presented in reaction (26).



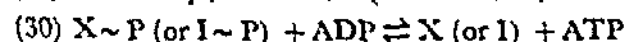
According to this scheme, there are three separate phosphorylative steps in the respiratory chain, in addition to the so-called "substrate-linked" step in the α-ketoglutarate chain.

The mechanism of these three phosphorylative steps in the respiratory chain is not known. Our current formulation, which is consistent with all the experimental findings, is expressed in general terms by equations (27)-(30)

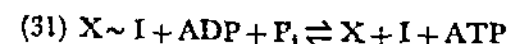

(27) $AH_2 + B + I \rightleftharpoons A \sim I + BH_2$


(28) $A \sim I + X \rightleftharpoons X \sim I + A$


(29) $X \sim I + P_i \rightleftharpoons X \sim P + I$ (or $I \sim P + X$)


(30) $X \sim P$ (or $I \sim P$) $+ ADP \rightleftharpoons X$ (or I) $+ ATP$

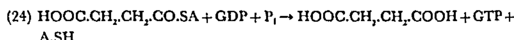
According to this scheme, the energy which can be liberated in the oxido-reduction reaction between two members of the respiratory chain (AH_2, hydrogen donor; B, hydrogen acceptor) is conserved in the high-energy compound $A \sim I$. In reaction (28) the energy is transferred to form the intermediate $X \sim I$ and the component of the respiratory chain (A) is liberated. In reactions (29) and (30), the sum of which is reaction (31), the energy of $X \sim I$ is utilized to form ATP.


(31) $X \sim I + ADP + P_i \rightleftharpoons X + I + ATP$

It is supposed that the three different steps in the respiratory chain have three different I's (I_1, I_2 and I_3) and three different enzymes (or, more likely, enzyme systems) catalyzing the appropriate reaction (31). Studies of the effect of pH on the ATPase activity, in the presence of DNP, and on the oxidative phosphorylation have, in fact, given evidence for the presence of three different reactions (31), with pH optima at 6.3, 7.4, and 8.5, respectively (Myers and Slater, 1957; Hülsmann and

oxidized by α-lipoic acid (thioctic acid) in reaction (22). The products of this reaction, acetic acid and reduced lipoic acid, remain bound together in the form of a thiol ester. In reaction (23), the acetyl group is transferred from the thiol group of α-lipoic acid to coenzyme A, liberating free reduced α-lipoic acid, which is then oxidized by DPN in the second step of the hydrogen-transfer chain.

The first hydrogen-transfer step of reaction (16) follows a similar course with the formation of succinyl-CoA. Free succinic acid is liberated by reactions (24) and (25) and then enters reaction (17),



(24) $HOOC.CH_2.CH_2.CO.SA + GDP + P_i \rightarrow HOOC.CH_2.CH_2.COOH + GTP + A.SH$



(25) $GTP + ADP \rightarrow GDP + ATP$

Two separate flavoproteins are required, the first (fp_I) *for the oxidation of DPNH and the second* (fp_{II}) *for the oxidation of succinate.*

The exact role of cytochrome b in the respiratory chain is doubtful. It is probably involved in the oxidation of succinate, but its function in the oxidation of the chains containing DPN is doubtful. Additional probable (cytochrome c_1) and possible (e.g. α-tocopherol) components of the respiratory chain have been omitted from the above schemes for reasons of simplicity.

The oxidation of isocitrate by heart appears to require both TPN and DPN. The pyridine nucleotide transhydrogenase, which catalyses the reaction between TPNH and DPN, is particularly active in heart.

3. *Oxidative Phosphorylation*

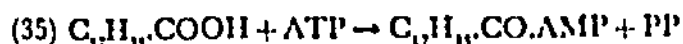
Each of the five oxidative steps involved in the oxidation of pyruvic acid is coupled with phosphorylation. There appear to be three phosphorylation steps in reactions (10), (14), and (19), four in reaction (16), and two in reaction (17), giving a total of fifteen. Ignoring for the moment possible side reactions which lead to loss of ATP, it can be concluded that the oxidation of pyruvic acid is accompanied by the synthesis of 15 molecules of ATP.

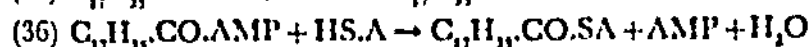
The mechanism of synthesis of one of these ATP molecules, the extra phosphorylation in reaction (16), has already been described in reactions (24) and (25). This phosphorylative step does not occur in the analogous reaction (10) because in this case the acetyl-CoA is used to synthesize citrate by reaction (11).

Much less is known about the mechanism of synthesis of the other

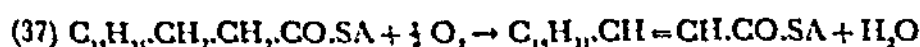
dria. Much of the recent work on this mechanism has, in fact, been carried out with enzymes prepared from heart muscle.

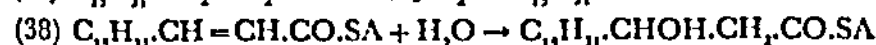
Fatty acids, such as stearic acid, must first be "activated" by formation of an acyl-CoA compound before they can be oxidized. The activation reaction is described by equations (35) and (36).

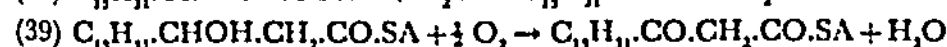

(35) $C_{17}H_{35}.COOH + ATP \rightarrow C_{17}H_{35}.CO.AMP + PP$

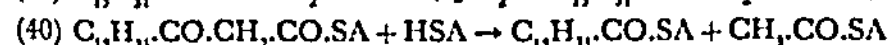

(36) $C_{17}H_{35}.CO.AMP + HS.A \rightarrow C_{17}H_{35}.CO.SA + AMP + H_2O$

The activated fatty acid is then oxidized to acetyl-CoA by a repeating "spiral" (the Lynen spiral) of four reactions, each turn of the spiral taking place with a fatty acid containing two carbon atoms less than in the preceding turn. The first turn of the spiral is described by equations (37)–(40).


(37) $C_{15}H_{31}.CH_2.CH_2.CO.SA + \frac{1}{2} O_2 \rightarrow C_{15}H_{31}.CH{=}CH.CO.SA + H_2O$


(38) $C_{15}H_{31}.CH{=}CH.CO.SA + H_2O \rightarrow C_{15}H_{31}.CHOH.CH_2.CO.SA$


(39) $C_{15}H_{31}.CHOH.CH_2.CO.SA + \frac{1}{2} O_2 \rightarrow C_{15}H_{31}.CO.CH_2.CO.SA + H_2O$

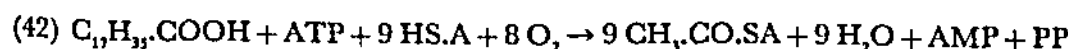

(40) $C_{15}H_{31}.CO.CH_2.CO.SA + HSA \rightarrow C_{15}H_{31}.CO.SA + CH_3.CO.SA$

Thus, each turn of the spiral yields one molecule of acetyl-CoA and one molecule of a fatty acid acyl-CoA derivative which contains two carbon atoms less than the compound at the beginning of the spiral. After eight complete turns, the reaction corresponding to equation (40) is equation (41).

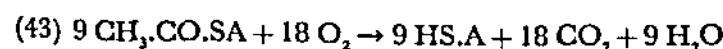

(41) $CH_3.CO.CH_2.CO.SA + HSA \rightarrow CH_3.CO.SA + CH_3.CO.SA$

This last reaction is exceptional in that two molecules of acetyl-CoA are produced.

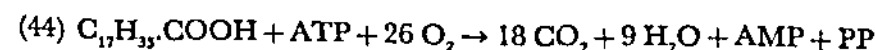
The complete operation of the Lynen spiral on stearic acid yields the sum reaction (42).


(42) $C_{17}H_{35}.COOH + ATP + 9\,HS.A + 8\,O_2 \rightarrow 9\,CH_3.CO.SA + 9\,H_2O + AMP + PP$

The acetyl-CoA can then enter the Krebs cycle [reaction (43)].

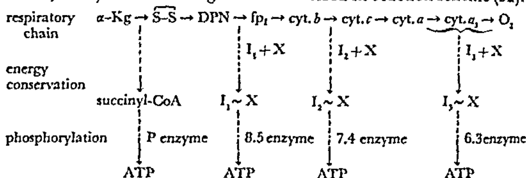

(43) $9\,CH_3.CO.SA + 18\,O_2 \rightarrow 9\,HS.A + 18\,CO_2 + 9\,H_2O$

The sum of equations (42) and (43) is equation (44).

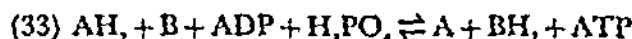

(44) $C_{17}H_{35}.COOH + ATP + 26\,O_2 \rightarrow 18\,CO_2 + 9\,H_2O + AMP + PP$

It should be noted that ATP is necessary only once, in the initial "priming" or "activation reaction" [equations (35) and (36)]. This initial priming is necessary for the formation of the first thiol ester,

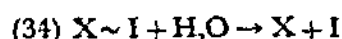
Slater, 1957). These findings are summarized in reaction scheme (32).



The sum of reactions (27)–(30) is reaction (33).


(33) $AH_2 + B + ADP + H_3PO_4 \rightleftharpoons A + BH_2 + ATP$

This shows that for each pair of hydrogen atoms transferred, one molecule of ATP is synthesized, i.e. P/2 H = 1.0. This means that the P:O ratio for the oxidation of various substrates must equal the number of phosphorylative steps involved in the oxidation of each substrate, i.e. 4.0 for α-ketoglutarate, 3.0 for isocitrate, malate, pyruvate, and 2.0 for succinate. In fact, the experimentally determined P:O ratios with isolated sarcosomes are considerably below these values. Aging of the sarcosomes or the addition of "uncoupling" agents such as DNP cause even lower P:O ratios. To explain these findings, it is assumed that X ~ I can react with water, according to reaction (34).


(34) $X \sim I + H_2O \rightarrow X + I$

Thus, reactions (29) and (34) will compete for X ~ I. Both reactions bring about the liberation of X and I, thereby allowing reactions (27) and (28) to proceed, but only reaction (29) causes the formation of ATP. It is supposed that aging of the mitochondria, or the addition of DNP, is able in some unspecified manner to increase the velocity of reaction (34). A substance soluble in organic solvents which can be extracted from aged mitochondria appears to play some role in the development of reaction (34) during aging of the mitochondria (Hulsmann *et al.*, 1958).

B. Oxidation of Fatty Acids

The mechanism of the oxidation of fatty acids has been studied with preparations obtained from various tissues. Since there is no evidence of tissue specificity, it will be assumed in what follows that the mechanism is the same in muscle sarcosomes as in, for example, liver mitochon-

lyzed by these sarcosomes (Slater and Holton, 1954; Holton, 1954).

(49) Asp. + α-Kg	→ OxAc + glut.
(50) α-Kg + DPN^+ + ADP + P_i	→ Succ. + CO_2 + DPNH + H^+ + ATP
(51) OxAc + DPNH + H^+	→ Mal. + DPN^+
Sum (52) Asp. + 2 α-Kg + ADP + P_i	→ Succ. + CO_2 + Glut. + Mal. + ATP

The transaminases contain pyridoxal as a prosthetic group. The pyridoxal acts as an amino-transferring intermediate, pyridoxamine being formed in the process.

IV. ENERGY-CONSUMING REACTIONS IN SARCOSOMES

Little is known about possible energy-consuming reactions in the sarcosome. Like mitochondria in other tissues, sarcosomes are structurally unstable in the absence of ATP. This suggests that some energy, in the form of ATP, is required to stabilize the structure. ATP is also required to maintain a difference in concentration of certain ions between the sarcosome and the medium.

In the liver, the microsome plays an important part in energy-consuming reactions, such as fat and protein synthesis. There is little evidence from differential centrifugation that heart muscle contains microsomes (Slater, 1957), although the endoplasmic reticulum, which is believed to be the structure in the intact liver cell which gives rise to microsomes when the cell is homogenized, has been identified in muscle cells by electron microscopy (Palade, 1955). It appears possible, then, that in muscle the sarcosome carries out some of the functions performed by the microsome in other tissues. But more work is required to settle this point.

Simpson and McLean (1955) have reported that 1-^{14}C-leucine and 3-^{14}C-phenylalanine were incorporated, *in vivo*, equally rapidly into the protein of a sarcosome or a microsome fraction isolated from rat skeletal muscle. McLean *et al.* (1956) have reported similar results from experiments *in vitro*.

V. PROPERTIES OF ISOLATED SARCOSOMES

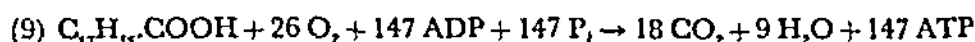
A. Isolation of Sarcosomes

Both morphological and biochemical criteria have been used in comparing different procedures for the isolation of sarcosomes. Morphologically intact sarcosomes appear under the visual microscope as small

which can then be oxidized by equations (37) and (39) of the spiral to the β-ketoacyl-CoA compound. These oxidation reactions introduce sufficient energy into the molecule, so that further formation of thiol esters can proceed by reaction (40) without the intervention of ATP.

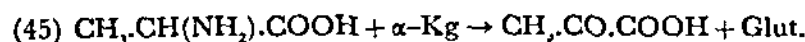
In fact, it must be expected that the two oxidation reactions of the Lynen spiral, which require the same sort of respiratory chain as the Krebs cycle, must be accompanied by the synthesis of ATP, although the author is not aware that this has been directly demonstrated. An experimental difficulty is that long-chain fatty acids, by virtue of their surface activity, cause structural damage to mitochondria.

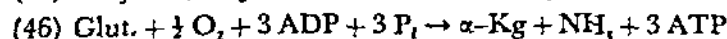
Reactions (37) are carried out by a series of flavoproteins, acyl-CoA dehydrogenases, which resemble the flavoprotein succinic dehydrogenase. The reaction, the formation of $-CH = CH-$ from $-CH_2.CH_2-$, is also very similar to that catalyzed by succinic dehydrogenase. For this reason, it can be tentatively assumed that 2 molecules of ATP are synthesized in each reaction (37) of the spiral, i.e. 16 in the 8 turns of the spiral. Reaction (39) requires a typical DPN-reacting dehydrogenase of the same type as malic dehydrogenase and would be expected to yield 3 molecules of ATP, i.e. 24 in total. Thus reaction (42) would give a net yield of $16 + 24 - 1 = 39$ molecules of ATP. Reaction (43) yields 108 molecules of ATP, so that the total oxidation of stearic acid is described by equation (9).


(9) $C_{17}H_{35}.COOH + 26\ O_2 + 147\ ADP + 147\ P_i \rightarrow 18\ CO_2 + 9\ H_2O + 147\ ATP$

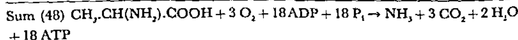
C. Oxidation of Amino Acids

Sarcosomes contain an active glutamic dehydrogenase (Paul *et al.*, 1952) and transaminase. Alanine, for example, is oxidized according to reactions (45)–(47).


(45) $CH_3.CH(NH_2).COOH + \alpha\text{-}Kg \rightarrow CH_3.CO.COOH + Glut.$


(46) $Glut. + \frac{1}{2}\ O_2 + 3\ ADP + 3\ P_i \rightarrow \alpha\text{-}Kg + NH_3 + 3\ ATP$


(47) $CH_3.CO.COOH + 2\frac{1}{2}\ O_2 + 15\ ADP + 15\ P_i \rightarrow 3\ CO_2 + 2\ H_2O + 15\ ATP$


Sum (48) $CH_3.CH(NH_2).COOH + 3\ O_2 + 18\ ADP + 18\ P_i \rightarrow NH_3 + 3\ CO_2 + 2\ H_2O + 18\ ATP$

The glutamic oxidase [reaction (46)] is a typical pyridine-nucleotide-requiring system, like reaction (19).

The presence of aspartic acid transaminase in rat heart sarcosomes is responsible for an anaerobic phosphorylation reaction which is cata-

one-half of the sarcosomes by gently grinding by hand in a mortar with coarse sand for about 2 minutes. The isolation medium was either 0.115 *M* KCl, 0.02 *M* phosphate, 0.01 *M* EDTA, pH 7.4; or 0.28 *M* sucrose, 0.01 *M* EDTA, pH 7.4. After dilution with the isolation medium, the myofibrils, erythrocytes, and nuclei were sedimented together with sand by a low-speed centrifugation, followed by a high-speed centrifugation to sediment the sarcosomes. Other workers have used homogenizers of the Potter-Elvehjem type to disintegrate the heart muscle.

Chappell and Perry (1954) found that the best results with pigeon breast muscle were obtained with a medium containing 0.05 *M* TRIS buffer, 0.001 *M* ATP, 0.005 *M* $MgCl_2$, 0.001 *M* EDTA, pH 7.4. Holton *et al.* (1957) also obtained somewhat better preparations of rat heart sarcosomes, when ATP was included in the isolation medium. The other additions suggested by Chappell and Perry (1954) had no significant effect with rat heart sarcosomes.

EDTA was first used for the isolation of sarcosomes by Slater and Cleland (1952). The addition of this chelating agent has no detectable effect on the properties of the freshly isolated sarcosomes, provided that the temperature does not rise above 5°C. during the isolation procedure, but has a very marked effect on the stability of the sarcosomes (both morphologically and biochemically) at room temperature (Slater and Cleland, 1953; Slater, 1957). EDTA prevents the binding by the sarcosomes of calcium during the isolation procedure. Other Ca^{++}-binding agents such as ATP and serum albumin are also effective. There is, therefore, no need to add EDTA when, for other reasons, ATP or serum albumin are added to the isolation medium. The addition of low concentrations of EDTA (10^{-3} *M*) in reaction mixtures is, however, often desirable to bind contaminating heavy metals, which have damaging effects on the structure of the sarcosome. No deleterious effects of neutralized EDTA, in this concentration, have yet been found.

The present procedure used in the author's laboratory for isolating sarcosomes from rat or guinea pig heart is as follows (Greengard *et al.*, 1959).

A rat or guinea pig is killed by a blow on the head and the heart quickly removed. Auricular and connective tissue and fat are removed and the heart placed in an ice-cold solution of 0.23 *M* sucrose, 0.005 *M* ATP, pH 7.4. The solution is decanted and replaced by fresh solution. The heart is finely minced with scissors and, in 2–3 batches, is homoge-

spheres. Damage is revealed by swelling, usually accompanied by other microscopically detectable changes of structure which are discussed later. These swollen sarcosomes sediment in the centrifuge rather more slowly than do the intact granules.

The following biochemical criteria are considered to be associated with undamaged sarcosomes: (a) high rate of respiratory activity in the presence of phosphate acceptor; (b) high rate of oxidative phosphorylation (P: O ratio); (c) dependence of respiration on presence of phosphate acceptor (respiratory control); (d) low ATPase activity in the presence of magnesium, and absence of DNP or other reagents which uncouple phosphorylation from respiration.

1. *Insect Thoracic Muscle*

Sarcosomes were first separated from insect muscle by Kölliker (1888) more than fifty years before the first isolation of liver mitochondria. In the procedures used by Watanabe and Williams (1951) and by most subsequent workers, the thoraces are gently pounded (without grinding action) with a small volume of isolation medium. The mash is then diluted with isolation medium and filtered through muslin to remove fibrils and pieces of wing. The sarcosomes are collected by a low-speed centrifugation and resuspended in the isolation medium.

Lewis and Slater (1954) found that 0.21 *M* sucrose, containing 0.01 *M* EDTA adjusted to pH 7.4, was the most satisfactory isolation medium from the point of view of high P: O ratios with sarcosomes isolated from the blowfly *Calliphora erythrocephala*. Sacktor and Cochran (1957c) also use 0.25 *M* sucrose containing EDTA (0.005 *M*, adjusted to pH 7.3) for the isolation of sarcosomes from the housefly *Musca domestica*[7]. The addition of ATP to the isolation medium, which has been found beneficial in the case of rat heart and pigeon breast sarcosomes, has not yet been tried with insect sarcosomes.

2. *Mammalian Muscle*

The sarcosomes are much more difficult to isolate from between the myofibrils of heart muscle. Cleland and Slater (1953a) liberated about

[7] Sacktor (1954) had earlier reported that the inclusion of EDTA in the isolation medium (0.25 *M* sucrose) completely inhibited the respiration of these sarcosomes. Since Sacktor and Cochran (1956, 1957a) now include neutralized EDTA in the isolation medium, it appears likely that the earlier results were due to the failure to neutralize the EDTA. Rees (1954) has also found increased P:O ratios with the sarcosomes of *Locusta migratoria* by the addition of EDTA.

sarcosomes are less damaged, since it probably merely reflects the higher concentration of the cytochromes in the sarcosomes (Table VI).

(b) The P:O ratio of our preparations of heart sarcosomes, particularly with succinate as substrate, is appreciably less than that obtained with liver mitochondria [see, however, Hatefi and Lester (1958)]. However, because of the greater respiratory activity, the rate of phosphorylation (given by Q_P in Table VI) is appreciably greater in sarcosomes.

TABLE VI

ENZYMATIC ACTIVITIES OF LIVER MITOCHONDRIA AND HEART SARCOSOMES[a]

	Rat liver mitochondria (I)	Heart sarcosomes (II)		II/I	
		Rat	Guinea pig	Rat	Guinea pig
α-Ketoglutaric oxidase					
Q_{O_2}	30	148		4.9	
P:O	3.06	2.86		0.97	
Q_P	183	850		4.6	
Succinic oxidase					
Q_{O_2}	175	327		1.9	
P:O	1.50	0.95	1.26	0.63	0.84
Q_P	525	620		1.2	
Cytochrome $(a+a_3)$				6.1	
Cytochromes $b+c+c_1$				2.7	
Respiratory-control index	7.0	4.8		0.7	
ATPase activity[b]	11.9	54.0		4.5	
r[c]	20	2.0	3.0	0.1	0.15

[a] The results for α-ketoglutaric oxidase, respiratory-control index, and ATPase [illegible] /mg. protein/hr.

[b] In presence of 0.003 M $MgCl_2$ and 10^{-4} *M* DNP.

[c] $r = \frac{\text{ATPase in presence of } 10^{-4}\ M \text{ DNP and } 0.003\ M\ MgCl_2}{\text{ATPase in presence of } 0.003\ M\ MgCl_2}$

These values are calculated from the means of the values for 1/r given in Fig. 2 of Greengard *et al.* (1959).

nized for 10–15 seconds at moderately high speed in a Potter-Elvehjem homogenizer fitted with a Teflon pestle and containing 5–10 ml. of the sucrose-ATP solution. The homogenizer is immersed in ice-water during this operation. The homogenate from each batch of heart is poured into cold centrifuge tubes and the combined homogenates centrifuged for 3 minutes at 600 *g* at 0–2°C. The supernatant is decanted into other centrifuge tubes, without contamination by the sediment, and centrifuged for 15 minutes at 600 *g* at 0–2°C. The supernatant is completely decanted and the sediment suspended in an ice-cold solution of 0.24 *M* sucrose, 0.001 *M* ATP, pH 7.4, with the help of the homogenizer (hand-operated).

B. Properties of Isolated Sarcosomes

1. *Mammalian Muscle*

Tables V and VI compare the composition and enzymatic activities, respectively, of our preparations of rat heart sarcosomes and rat liver mitochondria.

Considering the biochemical criteria of undamaged mitochondria listed above:

(a) The sarcosomes have a considerably higher respiratory activity than liver mitochondria. This should not be taken to indicate that the

TABLE V

COMPOSITION OF RAT LIVER MITOCHONDRIA AND RAT HEART SARCOSOMES[a]

	Liver mitochondria	Heart sarcosomes
Lipid (per cent)	30[b]	28
Phospholipid (per cent)	18[b]	24
DPN (μmoles/g. protein)	4.5	6.4
Total "adenosine" [c] (μmoles/g. protein)	—	38
"Energy-rich" phosphate (μmoles/g. protein)	—	20
Calcium (μmoles/g. protein)	61, 11[d]	99
Magnesium (μmoles/g. protein)	68[d]	290
Iron (μmoles/g. protein)	22	18

[a] Except where stated, the values are taken from Slater (1957) or Holton *et al.* (1957).

[b] Ada (1949) (rabbit).

[c] Calculated from absorbance at 260 mμ.

[d] Thiers and Vallee (1957) (perfused rat liver used for preparation of mitochondria).

of the blowfly by a very mild procedure give even lower ratios (see below). A final answer to the question whether the important difference between our preparations of heart sarcosomes and liver mitochondria represents a real difference *in vivo*, or is purely caused by mechanical damage or some other changes during the isolation of the sarcosome, may be possible when more is understood about the way mechanical damage or aging of the mitochondria promote the hydrolytic reactions.

It is interesting to compare the maximum respiratory activity of isolated sarcosomes with the consumption of oxygen by the heart *in vivo*. Since a large part of the oxygen uptake involved in the oxidation of fatty acids and amino acids occurs in the Krebs cycle, the respiratory activity of the sarcosome is best expressed by its activity with pyruvate as substrate, in the presence of oxaloacetate (or other Krebs cycle intermediate). Some measurements with different animals are summarized in Table VII. Sarcosomes isolated from animals as far apart in size as the ox, the guinea pig, and the rat have very similar activities.

The concentration of sarcosomes in the heart can be calculated if it is assumed that (a) all the cytochrome oxidase in the heart muscle is

TABLE VII

RATE OF OXIDATION OF PYRUVATE BY ISOLATED HEART SARCOSOMES[a]

Animal	Temperature °C.	Q_{O_2} (observed)	Q_{O_2} (corrected)	Reference
Rat	27°	138	276	Montgomery and Webb (1956)
	37°	290	290	Hulsmans (unpublished)
Guinea pig	30°	48	79	Plaut and Plaut (1952)
	37°	289	289	Hulsmans (unpublished)
Ox	30°	162	266	Hatefi and Lester (1958)

[a] The Q_{O_2} (corrected) was calculated for 37°C., assuming that the rate of the reaction doubled for a 10° rise in temperature.

present in the sarcosomes; (b) there is no loss of this enzyme in the isolation procedure; (c) isolated sarcosomes are uncontaminated by other fractions in the cell. Suppose that 1 g. heart contains *P* mg. protein, that the Q_{O_2} (cytochrome oxidase) of the homogenate of the whole heart muscle is Q_1 and that of the isolated sarcosomes is Q_2. Then 1 g. heart contains $P \times Q_1/Q_2$ mg. sarcosomal protein. These calculations have been made in Table VIII. The results vary between 19.5 and

(c) The respiratory-control index (rate of respiration in presence of phosphate acceptor/rate in absence of phosphate acceptor) is rather greater with liver mitochondria than with heart sarcosomes.

(d) The stimulation of ATPase activity (in the presence of Mg^{++}) by the addition of DNP is much less in the case of heart sarcosomes than in the case of liver mitochondria. The ATPase activity (in the presence of DNP) is 4.5 times higher in heart sarcosomes than in liver mitochondria. This is probably directly related to the higher concentration of phosphorylating enzymes (i.e. Q_P in oxidative phosphorylation) in the sarcosomes.

One of the most important differences between isolated sarcosomes and liver mitochondria is the ability of the former to hydrolyze added ATP in the presence of Mg^{++}. This reaction was first shown by Kielley and Meyerhof (1948) in granules isolated from rat leg muscles. On the other hand, isolated liver mitochondria have no ATPase activity, even in the presence of Mg^{++}, unless DNP is added (Kielley and Kielley, 1951). In the presence of DNP, it is not necessary to add Mg^{++}.

The ATPase of isolated sarcosomes has been studied by Sacktor (1953) and Sacktor and Cochran (1957c) in *Musca domestica*, by Chappell and Perry (1953, 1954) in pigeon breast muscle and by Holton *et al.* (1957) in heart muscle. All preparations have a high activity in the presence of added Mg^{++}, and show varying degrees of stimulation on the further addition of DNP. The heart sarcosomes also have a high activity in the absence of Mg^{++} when DNP is added. These properties of freshly prepared sarcosomes are similar to those of partly aged liver mitochondria.

We explain the difference between freshly isolated heart sarcosomes and liver mitochondria by the ability of the former preparations to bring about reaction (34) (the hydrolysis of $X \sim I$) (Slater, 1953; Myers and Slater, 1957; Purvis and Slater, 1959) or, possibly, of $X \sim P$. The P: O ratio of various types of mitochondrial preparations is quantitatively related to the ATPase activity (Greengard *et al.*, 1959).

With respect to the greater velocity of the hydrolytic side reactions, isolated heart sarcosomes resemble partly damaged liver mitochondria. The fact that the heart muscle which is most readily homogenized, namely that from young guinea pigs, usually gives the lowest ATPase activity and the highest P: O ratios with succinate (Table VI) suggests that the rat heart sarcosomes might be mechanically damaged during their isolation. However, sarcosomes isolated from the thoracic muscle

the same in both cases (Chappell and Perry, 1953; Cleland and Slater, 1953b). Thus, isolated sarcosomes from pigeon breast muscle could account for about 3.6 ml. O_2/100 g. muscle per minute. The actual oxygen consumption, *in vivo*, is not known to the author.

Each milliliter of O_2 consumed in the oxidation of pyruvate or fat can bring about the synthesis of about the same amount of ATP, viz. 268 µmoles (ignoring hydrolytic side reactions). Therefore, a mammalian heart is capable of synthesizing about 3.5 mmoles ATP per 100 g. heart per minute. Under the concentration conditions in the cell, the hydrolysis of a molecule of ATP makes available about 15,000 cal. of free energy (Burton and Krebs, 1953). Thus the sarcosomes in 100 g. heart make available about 50 cal. of free energy per minute.

The yield of ATP per milliliter O_2 may be compared with the relationship between the work done by the dog heart and the O_2 usage. The results of Evans (1918) show that at maximum efficiency, an O_2 consumption of 330 ml. per hour is obtained with a work output of 150 kg.m./hr. This O_2 consumption is equivalent to the production of $268 \times 330 \times 10{,}000 \times 10^{-6}$ cal. $= 885$ cal. of free energy in the form of ATP. The work done $= 150 \times 2.4$ cal. $= 360$ cal. Thus the heart is about 41% efficient in converting the free energy arising from the hydrolysis of ATP into mechanical work. It should be noted that this calculation of the efficiency is different from that customarily made by the physiologist, who calculates the total energy available in the oxidation of foodstuffs, not just that portion which is used to synthesize ATP.

2. *Insect Thoracic Muscle*

A surprising property of isolated insect sarcosomes is that despite their great ease of isolation, they have all the properties of aged liver mitochondria. According to the criteria listed on p. 124, isolated insect sarcosomes are more damaged than heart muscle sarcosomes. The available evidence does not allow us to state with certainty whether this represents a real difference in the properties of the different mitochondria *in vivo*, or is caused by damage during the isolation procedure. It is, however, difficult to reconcile the low respiratory activity of isolated sarcosomes with the high mechanical activity of the wing muscle in the fly (see below).

The relatively low respiratory activity, the small amount of material available for the biochemist, and low P: O ratios at first gave difficulties

39 mg. sarcosomal protein per gram of heart. The mean of the values given in Table VIII (28.3) will be used in the subsequent calculations.

Excluding Plaut and Plaut's (1952) value for guinea pig sarcosomes,

TABLE VIII

CONCENTRATION OF SARCOSOMES IN HEART MUSCLE

Animal	P[a]	Q_1	Q_2	$C = P\frac{Q_1}{Q_2}$	Reference
Rat	141	360	1300	39	Cleland and Slater (1953b)
Rabbit	98	296	1110	26.1	Slater (unpublished)
Cat	141	220	1590	19.5	Cleland and Slater (1953b)
Horse	180	132	835	28.5	Bouman and Slater (1957)[b]

[a] P = protein content (mg./g.) of heart muscle,
$Q_1 = Q_{O_2}$ (μl. O_2/mg. protein/hr.) of homogenate,
$Q_2 = Q_{O_2}$ of isolated sarcosomes,
C = sarcosome content of heart (mg. sarcosomal protein/g. heart).
[b] sarcosomal fragments prepared.

which is considerably lower than the others, the mean Q_{O_2} in Table VII is 280. Thus, the respiratory activity per gram of heart is:

$$\frac{28.3 \times 280 \times 100}{1000 \times 60} = 13.2 \text{ ml. } O_2/100 \text{ g. heart/min.}$$

This value may be compared with the following figures, given in the same units, for the oxygen utilization by the heart *in vivo*: human, 9.7 (Bing *et al.*, 1954); dog, 9.7 (Goodale *et al.*, 1948). [These values may be compared with the maximum consumption of O_2 by the working leg muscles of man measured by Asmussen *et al.* (1939), viz. 11.2 ml. O_2/100 g. working leg muscle/min.]. Thus, it can be concluded that isolated heart sarcosomes have sufficient respiratory activity to account for the oxygen utilization by the heart *in vivo*. Furthermore, the results suggest that the sarcosomes, *in vivo*, are working near their full capacity.

The respiratory activity of sarcosomes isolated from pigeon breast muscle is appreciably lower. Chappell and Perry (1953) found a Q_{O_2} with pyruvate of 46.5 at 30°C. (corresponding to about 76 μl. O_2 per milligram protein per hour at 37°C). Although it is not possible to calculate the concentration of sarcosomes per gram of heart in the same way as in Table VIII, it appears likely that the values for heart muscle and pigeon breast muscle will not differ greatly, since the proportion of the total nitrogen of the muscle which is found in the sarcosomes is about

quency, there remains a very serious discrepancy between the observed O_2 consumption of the fly, *in vivo*, and the rates of O_2 uptake which have been obtained with isolated insect sarcosomes. Levenbook and Williams (1956) have calculated that the Q_{O_2} (on a dry weight basis) of the sarcosomes of *Drosophilia virilis*, *in vivo*, is 2000. This is very much higher than the Q_{O_2} of isolated sarcosomes. Thus, Sacktor (1955) found a Q_{O_2} with pyruvate of only 21.5 at 25°C. Lewis and Slater (1954) found that the mean Q_{O_2} (on a protein basis) of sarcosomes, isolated from *Calliphora erythrocephala* in sucrose-EDTA, oxidizing α-ketoglutarate was 13 at 25°C. This value could be approximately doubled by adding albumin (cf. Watanabe and Williams, 1953; Sacktor, 1954). Since this value is based on measurements of the disappearance of α-ketoglutarate, it represents the one-step oxidation of α-ketoglutarate to succinate. Even if it is assumed that this is the slowest step of the Krebs cycle, the Q_{O_2} for the oxidation of pyruvate cannot exceed 130, on a protein basis, or about 90, on a dry weight basis. Isolation in a saline medium gave a much higher Q_{O_2}, with poorer phosphorylation. The highest Q_{O_2} found by Lewis and Slater was 50 (protein basis) corresponding to a maximum Q_{O_2} (dry weight basis) for the oxidation of pyruvate of about 175. Rees has found rather higher Q_{O_2}'s (at 32°C.) for sarcosomes of *Locusta migratoria* (85 with α-ketoglutarate, 54 with glutamate, and 85 with malate).

There appear to be three possible explanations for the discrepancy between the high O_2 consumption of the fly, *in vivo*, and the rates of O_2 uptake with isolated sarcosomes: (a) the respiratory-enzyme systems of the isolated sarcosomes are damaged during isolation; (b) the isolated sarcosomes are not readily permeable to intermediates of the Krebs cycle (Lewis and Slater, 1954); (c) a large part of the respiration of insect sarcosomes, *in vivo*, occurs by a pathway different from that of the Krebs cycle. In this connection, the high activity of α-glycerol phosphate dehydrogenase in insect sarcosomes reported by Sacktor and Cochran (1957*a*, 1958), Chance and Sacktor (1958) and Estabrook and Sacktor (1958) is of interest (see, however, Gregg *et al.* (1959)).

This discrepancy remains a challenge to the insect physiologist and biochemist. The low P:O ratios usually obtained with isolated insect sarcosomes also deserve further investigation. The mean value found by Lewis and Slater with α-ketoglutarate as substrate was 1.2, compared with a value of about 3 obtained with heart sarcosomes (Slater and Holton, 1954; Holton *et al.*, 1957). Sacktor (1954) and Sacktor and

in detecting oxidative phosphorylation in insect muscle. Sacktor (1953) was unable to obtain oxidative phosphorylation with sarcosomes isolated from *Musca domestica* L., but succeeded later with an improved isolation medium (Sacktor, 1954). Lewis and Slater (1953, 1954), in the meantime, had obtained phosphorylation with sarcosomes isolated from *Calliphora erythrocephala* using an isolation medium containing EDTA. The P:O ratios obtained, however, were mostly considerably below those found with heart sarcosomes or liver mitochondria.

Sacktor (1954) showed that the addition of high concentrations of serum albumin increased the P:O ratio and this was confirmed by Lewis and Slater (1954). Albumin has a similar effect with aged mitochondria (Polis and Shmukler, 1957) and also with the substance soluble in lipid solvents isolated from aged mitochondria by Hülsmann *et al.* (1958). It appears possible that the poor phosphorylative ability of isolated insect sarcosomes may be due to the liberation of a similar material during the isolation of sarcosomes.

Keilin (1925) showed that the thoracic muscles of flying insects contained the highest concentration of cytochrome among all the organisms examined. He suggested that this high concentration was connected with the ability of the wing muscles of insects to produce very rapid contractions. He also showed that the concentration of cytochrome increased after hatching.

Levenbook and Williams (1956) have studied quantitatively the various changes in the flight muscle of the blowfly *Phormia regina* which occur during aging. They found that during the first week of adult life, the weight of the sarcosomes increased about 3-fold, but the number (6.7×10^{9} per fly) did not change. The concentration of cytochrome *c* in the sarcosome did not change from the original value (about 1.6% of the dry weight), but in consequence of the increased size, the amount of cytochrome *c* in the sarcosome and therefore in the whole muscle increased about 3-fold. The mature sarcoscome has a volume of $4.2\ \mu^3$ (about 10 times that of a liver mitochondrion) and comprises 32.6% of the muscle mass (dry weight). The wing frequency also doubled during the first week of adult life. Earlier, Lewis and Slater (1954) had shown that the P:O ratio of sarcosomes isolated from the blowfly *Calliphora erythrocephala* is low during the first week of life.

While the increased concentration of cytochrome and the increased efficiency of oxidative phosphorylation during the first week of adult life would appear to have some connection with the higher wing fre-

particles which constitutes the Keilin and Hartree heart muscle preparation consists of these sarcosomal fragments. This preparation contains the entire respiratory chain necessary for the oxidation of succinate and DPNH [and also TPNH in the presence of DPN (Purvis, 1958)], but they lack many of the dehydrogenases, coenzymes, and other enzymes found in the intact sarcosome. In particular, they are unable to couple the oxidation of succinate or of DPNH with phosphorylation. This suggested to us that the sarcosome was made up of an external membrane, composed of a lipoprotein complex (containing 40% lipid), bounding a "gel" of enzymes, coenzymes, and salts. When water enters the sarcosome, the gel dissolves to give a solution of proteins, coenzymes, and salts which are washed away when the stretched membrane is fragmented (cf. Hogeboom and Schneider, 1952).

Our crude picture of the internal structure of the sarcosome was drawn before the fine structure, as revealed by the electron micrographs of Palade (1952) and Sjöstrand (1953), was known. These studies confirmed the existence of an external membrane, and showed that the interior was also largely made up of membranes (or cristae) of a similar structure. This characteristic structure of mitochondria revealed by the electron microscope has, in fact, become one of the most important means of identification of mitochondria (Dempsey, 1956) and provides the final evidence that sarcosomes are the mitochondria of muscle. Chapman (1954) has published some beautiful pictures of insect muscle, which clearly show the internal structure of the sarcosome. Weinstein (1954), Kisch (1956), and Howatson (1956) have studied the fine structure of heart muscle, heart muscle, and pigeon breast muscle, respectively. Palade (1952) found that heart muscle mitochondria were particularly rich in the internal cristae, and since it was known that these mitochondria were particularly rich in cytochrome, he suggested that it was the internal cristae rather than the external membrane which was the site of the respiratory chain.

Palade's suggestion is not incompatible with our observations, since recent studies of swollen mitochondria in the electron micrograph (Watson and Siekevitz, 1956) show that the cristae move close to the external membrane in swollen mitochondria, and the two would not be resolved by light microscopy. Watson and Siekevitz's (1956) and Ball and Barnett's (1957) observations with the electron microscope provide strong support for the concept that respiratory-chain preparations obtained by fragmentation of mitochondria consist of mitochon-

Sanborn (1956) have reported ratios of 1.6–2.0 and 1.2 (at 25°C.), respectively. Rees (1954) found a P:O ratio of 2.0 with the sarcosomes of *Locusta migratoria* oxidizing α-ketoglutarate, but obtained higher ratios with other substrates (glutamate, 2.9; malate, 2.3). Related to the low P:O ratios of isolated insect sarcosomes are the lack of respiratory control (Sacktor, 1954) and the high ATPase activity in the presence of Mg^{++} (Sacktor, 1953).

VI. STRUCTURE OF SARCOSOMES AND LOCALIZATION OF ENZYMES WITHIN THE SARCOSOMES

Kölliker (1888) observed that when insect sarcosomes were immersed in water, they swelled greatly and were transformed to vesicular-shaped bodies with a fine but clearly defined membrane. The contents of the sarcosomes took the form of a half-moon lying at one side of the vesicle, and often appeared to be partly dissolved and sometimes indeed totally disappeared. Dilute acids and alkali also caused the sarcosomes to swell, without, however, causing the contents to dissolve.

Harman (1950) observed similar transformations of the contents of mitochondria from various tissues, but did not observe the external membrane.

Cleland and Slater (1953a, b) studied in some detail the transformations of structure of rat heart sarcosomes which occurred either upon suspending the sarcosomes in hypotonic media or upon incubating a suspension in isotonic medium (not containing calcium-chelating compounds) at room temperature for a short period. The changes observed were identical with those reported by Kölliker and the disappearance of the contents of the sarcosome upon swelling was interpreted in the same way, i.e. as solution of the sarcosomal contents as more water entered the interior of the sarcosome.

The final stage of the swelling process leads to the formation of a large vesicle, in which the only microscopically visible part which darkens with osmic acid is the membrane. These swollen sarcosomes do not burst like erythrocytes if left undisturbed. If, however, they are mechanically damaged, e.g. by shaking a suspension in a test-tube, they fragment into small osmiophilic particles. Since the only osmiophilic material before the fragmentation was the membrane of the swollen sarcosome, we suggested that these particles consist of fragmented sarcosomal membrane.

It is clear from its method of preparation that the suspension of

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drial membranes, but do not enable a distinction between external and internal membranes to be drawn.

It is, indeed, unlikely that the external membrane of mitochondria is the sole source of the particles in respiratory-chain preparations, since the yield of such preparations as that of Keilin and Hartree is much higher than the percentage of the total area occupied by the external membrane in electron micrographs. These preparations probably consist of membranous material derived from both the exterior and the cristae, but this leaves the question open whether both types of membrane contain the respiratory chain, or only one. There is one piece of evidence which favores the view that at least a part of the respiratory chain is in the external membrane, viz. that mitochondria can react quite rapidly with added reduced or oxidized cytochrome *c* which would not be expected to be permeable to a membrane of the ordinary type but which could react with a respiratory chain situated in a membrane.

Gamble and Lehninger (1956) have studied the enzymic properties of fragments of mitochondria obtained in different ways. They showed that the oxidase and ATPase activities and chemical composition were largely independent of the mode of fragmentation and of the particle size. This suggests that all the membranous material in mitochondria, both external and in the cristae, is of similar composition. Although mitochondrial fragments are usually completely devoid of phosphorylative activity, it is possible to obtain particles with a limited ability to carry out oxidative phosphorylation by disruption with digitonin (Cooper and Lehninger, 1956). Particles with this property have also been isolated by Green *et al.* (1957).

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CHAPTER IV

Endocrines and Muscle

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The topic, "endocrines and muscle," is somewhat diffuse in nature, partly because of the variety of the hormones and the complexity of their relationships, but mainly because in the study of individual hormones, the choice of muscle as an object of the study has frequently been merely incidental to other aspects of the problem at hand. Much of our information, in fact, relates to the effects of hormones not on muscle alone but on the "peripheral tissues," and although the muscles make up the greater part of the mass of these tissues, it cannot be said that the effects observed are necessarily related directly or exclusively to the muscles themselves. In other instances, a single muscle such as heart or diaphragm may be chosen for the sake of convenience in experiment, but the results obtained with these preparations may not always be applicable to muscles as a whole or even to other individual muscles. It will be clear from what follows that our knowledge of endocrine effects on muscles is scattered and fragmentary. Because of this, it has seemed convenient to divide the topic into two parts, related to general processes: one, endocrines in growth and development and two, endocrine effects on metabolism and function. The following sections comprise a general summary of the main lines of work in these two respects. It is not intended to be complete, but to serve as an introduction to a complex and as yet but lightly explored area of study, both in endocrinology and in muscle physiology.

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the thyroid also will not develop properly. Although it is interesting that inhibition of the thyroid of the chick embryo with thiourea has effects on muscle protein and RNA similar to the effects of decapitation, this may not be taken to mean that thyroid deficiency is directly or entirely responsible for these changes, since the production of pituitary growth hormone may depend upon adequate thyroid function. Hsieh *et al.* (1952) have reported that the injection of bovine growth hormone, in doses of 5 to 40 μg. over a 5-day period starting at day 13, or in one dose on day 13, results in increased body weight of the chicks at hatching. These observations indicate the practical possibility of a study of the effects of pituitary hormones, singly and in combination, on muscle development and composition in the decapitated chick embryo.

Hypophysectomy in very young animals does not bring about immediate cessation of growth but is followed by a period of decelerating growth. The most detailed study of animals hypophysectomized shortly after birth (6 days) has been made by Simpson and her colleagues in rats (Simpson *et al.*, 1950). The observations are mainly devoted to the changes in skeletal development, and the changes in body weight (reflecting muscle mass) are reported almost incidentally. Both thyroidectomy and hypophysectomy inhibit growth. The injection of growth hormone into the very young thyroidectomized animal brings about resumption of growth. Thyroxine also induces a resumption of growth in these animals, but there is further development and maturation as well. In a similar series of experiments, Scow (1951, 1953, 1954) has made observations on the composition of muscle in rats thyroidectomized at birth, or hypophysectomized at weaning. Growth was inhibited in both cases. The muscles of the thyroidectomized rats were relatively deficient in myosin and water-soluble protein; those of the hypophysectomized rat were small, but of normal composition. Treatment of the thyroidectomized rats with growth hormone brought about gain in weight and a marked increase in the collagen content of muscle, but the myosin remained low and the weight of muscle per unit of body weight was still subnormal. On the other hand, injection of growth hormone into hypophysectomized rats resulted in increase in body weight, in muscle weight proportional to body weight, and in myosin content. It was concluded that some thyroxine as well as growth hormone was required for the preservation of the normal composition of skeletal muscle during growth, or that thyroxine is required for "normal development" of the muscle.

I. ENDOCRINE CONTROL OF GROWTH AND DEVELOPMENT

A. Fetal, Embryonic, and Neonatal Growth

During fetal life in viviparous species, the endocrine organs of both mother and fetus may play a part in determining the continuing development of the fetus. Once the placenta is well developed, pregnancy may be maintained despite surgical removal of the pituitary. Fetal development appears to progress normally. The young may be somewhat smaller than usual at term, but they are viable and normal in appearance and proportion (Knobil and Caton, 1953). If rabbit fetuses are decapitated (hypophysectomized), thyroidectomized, or both decapitated and thyroidectomized *in utero*, the subsequent gain in body weight is nearly normal. In this case, the maternal contribution to the hormonal needs of the fetus may be the determining factor. No observations seem to have been made as yet on decapitated fetuses carried by hypophysectomized mothers, from which it might be learned whether the placenta alone is capable of supplying all the hormones necessary for normal growth and development. Relatively few observations have been made on the effects of administering hormones to pregnant females. It is reported that the injection of growth hormone during pregnancy in rats results in significant increases in the birth weight of the young (Engfeldt and Hultquist, 1953).

In oviparous animals, the principal source of hormones is the developing embryo itself. Love and Konigsberg (1958) have recently described the changes that occur in chick embryos in which the prosencephalon was removed at stages 10 to 12 (after 33 to 40 hours incubation), thus removing both primordia of the pituitary. By the twentieth day the operated animals lagged significantly behind the controls in body weight, in the weight of the leg muscles, and in the protein and ribonucleic acid content of the leg muscles. At the same time, the deoxyribonucleic acid content of the leg muscles of the operated embryos was higher than that of the controls. The authors report similar changes in the protein and RNA content of the leg muscles of chick embryos treated with thiourea, but without the accumulation of DNA noted in the decapitated embryos.

The part played by specific hormones in these alterations during embryonic life remains to be determined. There is evidence that the development of the adrenal cortex is impaired, and one can expect that

In summary, it is clear that both growth hormone and thyroxine *may play an important part in the growth and development* of muscle even from the period of embryonic life. The thyroid hormone very likely does not influence the growth of the tissue directly, since it is also indispensable for the normal production of growth hormone, but the thyroid hormone may have independent effects on muscle comparable, perhaps, to its effects on bone—that is, on the proper development, or "maturation," of the tissue. Further study, especially of the composition of individual muscles and of the correlation of composition with functional characteristics, is required to clarify the respective rôles of growth hormone and thyroxine in the growth of muscle.

2. *Insulin.*

It is well established that young animals do not grow and adult animals do not maintain nitrogen balance in the absence of the pancreas or in extreme insulin deficiency such as may be induced by alloxan. Growth hormone is also ineffective in diabetic animals unless insulin is given as well (Gaebler *et al.*, 1956; Milman *et al.*, 1951; Scow, 1957; Scow *et al.*, 1958). It is observed that depancreatized animals maintained on a constant dose of insulin increase their nitrogen retention in response to increasing doses of growth hormone but that the response to the larger doses of growth hormone is improved by increasing the amount of insulin given. From this, it may be concluded that extra insulin is required in order for growth hormone to exert its maximal effect. Insulin is therefore indispensable for normal growth and development. In recent years, it has even been suggested that insulin may itself be the primary "growth hormone" and that the action of pituitary growth hormone may be merely supporting or permissive, establishing conditions in which the growth-promoting action of insulin can be exerted maximally (Salter and Best, 1953; Lawrence *et al.*, 1954; Salter, *et al.*, 1957; Ketterer *et al.*, 1957). Salter and Best have shown that young hypophysectomized rats, treated daily with insulin and kept alive on a diet high in carbohydrate with liberal supplements of sugar water, gain weight, retain some nitrogen, and increase in length. The "growth" so induced is characterized by a large increase in body fat, and it is supported only by a large increase in food intake. The effect is very different from that of pituitary growth hormone, which may induce a gain in weight with no increase in food intake and with no deposition of fat. The growth-promoting effect of insulin has recently

B. Postnatal Growth

1. *Growth Hormone and Thyroxine*

In older animals, hypophysectomy and thyroidectomy bring about almost immediate cessation of growth. Growth hormone will induce resumption of growth in both types of operated animal, but thyroxine is effective only in the thyroidectomized animal, although it will augment the effects of growth hormone in the hypophysectomized animal. This relationship has been observed repeatedly. The dwarfism of thyroid deficiency appears to be due not only to lack of thyroid hormone itself but also to a large extent to an induced deficiency of growth hormone which is not elaborated and produced by the pituitary in sufficient amounts when thyroid hormone is lacking (Eartly and LeBlond, 1954).

Studies of the composition of the materials laid on in normal or hypophysectomized animals treated with growth hormone show that the gain in weight is largely due to increases in water and in protein, a large proportion of which must be muscle protein. The proportion of fat becomes smaller and the composition of the whole body tends toward that characteristic of the young animal. There have been few studies of the effects of growth hormone on the size of individual muscles or on the composition of muscle. The most detailed study of this kind is that of Greenbaum and Young (1953). In young rats treated with growth hormone, all organs but the heart and soleus muscle increased in size. The internal organs were relatively little affected, and the response of individual muscles varied widely. One of the more responsive muscles—quadriceps—was studied in detail by Gray and Young (1954). There were no changes in the proportions of sarcoplasm, myofibril, or collagen and elastin. The ATPase activity of the myofibrils per gram of tissue was less than normal. Bigland and Jehring (1952) studied the behavior of quadriceps in rats treated with 0.5 mg. of growth hormone per day for 3 weeks, after which time the muscle had increased 20 to 25% in size. Despite the increase in size, there was no increase in twitch tension or summation tension; the tension per gram of muscle was less than normal. There was a decrease in the tension developed during a tetanus, but no change in the time required for fatigue. Thus the bigger muscle of the hormone-treated rats was not stronger than normal. This is consistent with the observation that acromegalics, despite their huge muscles, are often not strong in proportion to their size.

were made, the effects have been observed in several other species, including man, and a number of properties of the protein-anabolic effect have been established. The injection of androgens induces nitrogen retention in the absence of the pituitary, adrenals, thyroid, or testis, and in female as well as in male animals. In the absence of the pancreas, androgens will not bring about nitrogen retention unless insulin is also administered (Sirek and Best, 1953). In this respect, the protein anabolic effect of androgens is like that of growth hormone in being dependent upon insulin for complete expression. The nitrogen retention induced by androgen differs from that induced by growth hormone in an important respect: if the injection of androgen is stopped, there is a prompt "rebound" of the nitrogen excretion, which exceeds the pretreatment levels. In the castrate or eunuchoid animal, therefore, the gain in nitrogen is not consolidated. In hypophysectomized animals treated with growth hormone, there may be a small loss of weight after cessation of treatment, but the major part of the weight gained is retained, and the animals usually do not exhibit a negative nitrogen balance. In the rat, a curious feature of the protein anabolic effect of androgens is that it lasts only for a short time (7–9 days) and then dies out despite continued treatment. When the injections are stopped, there is a period of negative nitrogen balance, followed again by a period of slight positive nitrogen balance, although no more hormone is being given.

The amounts of nitrogen retained by castrate animals under the influence of androgen are far greater than can be accounted for by the growth of the secondary sexual apparatus (prostate, seminal vesicles, etc.). The effect, therefore, is more general, and it is largely expressed in an increase in the size of the skeletal muscles. For a time, indeed, the impression was current that the protein-anabolic effect of the androgens was in fact fairly general. This may have been due to the accident that most of the early detailed observations were made on the rat, in which nearly all the muscles respond to androgen. For example, Kochakian and Tillotson (1956) have shown that in the castrate rat treated with androgen, some 29 different skeletal muscles increased in size in fair proportion to the general gain in body weight. More recent studies however, have shown that in other species the effect is not so uniform. For instance, in the castrate guinea pig, Scow and Roe (1953) and Kochakian *et al.* (1957; Kochakian and Tillotson, 1957; Kochakian and Cockrell, 1958) have shown that the muscles of the upper back,

been carefully studied by Wagner and Scow (1957) in tube-fed hypophysectomized rats. When the same amount of food (about 30% more food than required to maintain body weight) is received by untreated and insulin-treated hypophysectomized rats, no differences were seen in gain in body weight, or in the composition of the weight gain. In 22 days, the insulin-treated and the control rats gained 30 and 32 gm., respectively; the gains in body fat were 21.7 and 21.2 gm., and the gains in protein were 2.4 and 2.7 gm. By contrast, another group of rats tube-fed an amount just sufficient to maintain their body weight, and treated with growth hormone, gained 27 gm. The gain in protein was 4.1 gm. and there was a *loss* of 1.6 gm. of body fat. Wagner and Scow conclude that exogenous insulin acts as a growth stimulant in hypophysectomized rats only by increasing their food intake. Although it is indispensable for normal growth, insulin does not seem to be primarily a growth-promoting hormone.

3. *Androgens*

A relation of the testis to muscular development and strength has been recognized for many centuries, and in modern times has often been experimentally confirmed. Castrate or hypogonadal males often have smaller muscles than their normal counterparts. Conversely, in females under the influence of a virilizing agent, as for instance from an adrenal tumor producing androgen, the musculature is often unusually well-developed, "masculine" in character. This effect of androgens on the muscles has been said to be independent of the pituitary or the adrenal, since it occurs in hypophysectomized or adrenalectomized castrate animals treated with androgen. The effect, however, is much smaller than it is in castrates (Scow, 1952). If the hypophysectomized animal is treated both with growth hormone and with androgen, the effects are additive. The heart as well as the skeletal muscle may be affected by castration. It is reported that the size and force of the heart are decreased in the castrate rat, and that treatment with androgen restores the weight and strength of the heart muscle to normal and increases the concentrations of glycogen, phosphocreatine, and adenosine triphosphate to the normal level (Schumann, 1940).

The effects of androgens on muscle have been studied most closely in relation to the protein-anabolic effects of these hormones, which were first demonstrated in castrate dogs by Kochakian and Murlin (Kochakian, 1946). In the twenty-odd years since these observations

grows, that it loses weight about in proportion to the loss in body weight during starvation or after overdoses of thyroid hormone or cortisone, and that it increases in weight during treatment of the animals with growth hormone. The muscle therefore responds to nonandrogenic influences, and might therefore be considered to reflect a nonandrogenic, protein-anabolic, or "myotrophic" action of androgens or of weakly androgenic or nonandrogenic derivative steroids. Observations on a number of steroids seemed indeed to suggest that their effect on the weight of levator ani in castrate rats was not strictly related to their relative potencies as androgens, although the most potent androgens were also the most effective "myotrophic" agents. Hershberger *et al.* (1953) have extended and partly confirmed the observations of Gordan *et al.* in a study of the relative effects of 32 different steroids on the weights of levator ani, the ventral prostate, and the seminal vesicles in rats castrated at 21 days of age and treated for the next 7 days. A selection of their data is presented in Table I. It will be

TABLE I

EFFECTS OF ANDROGENS ON THE WEIGHT OF SEMINAL VESICLES, VENTRAL PROSTATE, AND LEVATOR ANI IN 21-DAY-OLD CASTRATE MALE RATS[a]

Treatment	Dose (mg.)	Body weight (g.)	Ventral prostate (mg.)	Seminal vesicles (mg.)	Levator ani (mg.)
Controls	—	65	9.7 ± 0.4	7.0 ± 0.3	12.2 ± 0.5
Testosterone propionate	0.7	67	112.1 ± 3.9	94 5 ± 5.4	34.2 ± 1.6
Testosterone	0.7	68	46.5 ± 4.6	17.8 ± 1.3	21.9 ± 2.0
19-Nortestosterone	0.7	66	19.7 ± 2.5	12.1 ± 1.7	21.6 ± 0.5
Methylandrostenediol	0.7	67	29.5 ± 1.7	11.6 ± 0.6	15.4 ± 0.9
Androsterone	0.672	69	69.9 ± 8.9	7.6 ± 0.5	15.5 ± 0.5

[a] Treatment for 7 days beginning with day of castration.

seen that testosterone and its propionate, most potent as androgens, are also most active on levator ani; that methylandrostenediol, a weak androgen, has an effect on levator ani about in proportion to its effect on the ventral prostrate, and that 19-nortestosterone has a somewhat greater effect on the muscle than it has, relatively, on the ventral prostate. These observations have been extended to other derivatives of 19-nortestosterone by other workers (e.g. Barnes *et al.*, 1954a, b; Saunders and Drill, 1957). Compounds which were not at all androgenic had no effect on any of the organs. From this, one might conclude that an effect

shoulders, head, and neck, and especially the temporal muscles and masseters, are much more markedly increased in size by androgens than the other muscles of the body. During a prolonged fast, all the muscles of the guinea pig lose weight to the same degree, but if large doses of testosterone are given, the temporals, masseters, and shoulder musculature lose weight to a much smaller degree than the others. These observations and their own experiments indicating that androgen has little or no effect on the size, weight, or composition of the thigh muscles in the castrate rat have led Scow and Hagan (1957) to the conclusion that androgens exert their effects mainly on those muscles which are concerned with the mating activities of an animal, or which may, one may add, have some other advantage as a secondary sex character. Thus the quantitative aspect of the protein-anabolic effect of the androgens would be related to the relative mass of muscle especially involved in the mating activities of the species. The rat, perhaps somewhat exceptionally, seems to involve his entire "muscular person" in these activities, but the guinea pig may be more typical in the emphasis on the muscles of the head, neck, and forequarters. In men, the noble carriage of the head upon the well-modeled column of the neck, the mass and breadth of the shoulders, and the depth of the muscled chest and upper back are classic attributes of virility. The bull provides another example. The wide jowls and massy shoulders of the tomcat are in marked contrast to the dapper elegance of the queen of the same breed.

Wainman and Shipounoff (1941) were the first to show that the muscles of the perineal complex in the rat (bulbocavernosus, ischiocavernosus, and levator ani) remain small or undergo marked atrophy after castration, and increase in size under treatment with testosterone. They suggested that changes in the weight of this complex of muscles might be used as an additional test for the androgenic effects of steroids. Confirming these observations, Gordan and his colleagues measured levator ani alone (for the sake of simplicity and of better quantitative measurement), and suggested that the increase in weight of levator ani in response to androgens and to derivative steroids might be used as a measure of the protein-anabolic effects of the steroids, in contrast to their purely androgenic actions as expressed in their effects on the ventral prostate and seminal vesicles (Eisenberg *et al.*, 1949; Eisenberg and Gordan, 1950, 1954). Their suggestion is based on the observations that levator ani increases in weight slowly after castration, as the animal

The muscular organ which is obviously under ovarian control is the uterus, which undergoes a remarkable increase in size at sexual maturity. Experiments in castrate females have shown that the uterine development at maturity is due to estrogen. Both the endometrium and the myometrium take part in the response to estrogen, but at the moment it is difficult to say whether the increase in the muscular component is directly under the control of the ovarian hormone. The uterus, in the early phases of stimulation with estrogen, is distended with fluid, and it has been shown that artificial distension of the unstimulated uterus in castrate females leads to a similar hyperplasia of the muscle (Burrows, 1949). More recent work on biochemical changes in the uterus during the first few hours of stimulation with estrogen reveals that there is, first, a rapid early incorporation of $C^{14}O_2$ into adenine, guanine, and uridine of the acid-soluble nucleotides, and second, a marked accumulation of ribonucleic acid (Jervell *et al.*, 1958). During this time, there is also a marked increase in the activity of a number of amino acid activating enzymes (McCorquodale and Mueller, 1958). The stimulation of systems associated with protein synthesis is clearly indicated, but since these observations were made on uterine segments, the question whether both the muscle and the endometrium are responding to estrogen is unresolved.

II. METABOLISM AND FUNCTION

A. Insulin

1. *Action of Insulin on Carbohydrate Metabolism in Muscle*

From the earliest work on the physiological effects of insulin, it was apparent that the hypoglycemic action of the hormone was fully evident in eviscerated or hepatectomized animals. This suggested at once that skeletal muscle, which constitutes a large part of the metabolizing tissue of such a preparation, was probably an important site of action of insulin. Early attempts to demonstrate such effects directly were inconclusive, however, mainly because it was not appreciated at that time how sensitive the glycogen of muscle is to the effects of convulsive seizures induced by hypoglycemia, to anesthetic agents such as ether and morphine, and to the trauma and anoxia then usually incident to removal of the muscles for analysis. Best *et al.*, in 1926, using the spinal eviscerated cat, first showed that a considerable proportion of the glucose which disappeared under the influence of insulin was deposited

on levator ani is not readily separable from an effect in some degree upon other organs of the genital apparatus. Dorfman and Shipley (1956) have collected data illustrating the considerable differential effects of a variety of androgens on a number of their target organs. These observations suggest that a given steroid exhibiting a more marked effect on levator ani than on the ventral prostate or seminal vesicles may be no less an androgen than another steroid exerting proportionate effects on all of the organs of the genital apparatus. Levator ani may therefore be of doubtful value as an indicator of the protein anabolic action as distinct from the androgenic effect of a given steroid. This conclusion is supported by the observation of Nimni and Geiger (1957) that in castrate rats on a nitrogen-free diet both testosterone propionate and 19-norethandrolone cause an increase in weight of levator ani as well as of the seminal vesicles.

One would not wish to deny a general effect of androgen on the muscles. At the same time, in many species, man probably included, the effect is most marked on certain muscles in the service, as Scow suggests, of the mating activities of the animal. In most cases, the relative mass of the muscles involved in this expression of a secondary sex character is great enough to account for nearly all the protein anabolic effect of the androgens. The temporary, or reversible, nature of the effect is also accounted for. In summary, one may say that the androgens have a modest effect on the size and strength of the muscles in general, a more marked effect in many species on a limited group of muscles, and a profound effect on some muscles associated intimately with the sexual apparatus itself, such as levator ani and other muscles of the perineal group.

4. *Estrogens*

The hormones of the ovary are not known to have any specific effects on the growth of the general musculature. The smaller size of female animals is attributable to an effect of estrogens on the epiphyses, bringing about early closure and cessation of continued growth, particularly of the long bones. The lighter musculature and lesser strength of the female may be determined by the lack of androgen. This is obviously not the whole story. Female animals in the wild and even in the civilized state, are strong, active, and remarkably durable. It is possible that a closer study of the structure and function of muscle in the female would reveal its distinctive characteristics, but such a study has not been made.

blood cells certainly do not require insulin for normal metabolism and do not exhibit any response to insulin. Insulin also does not affect the tubular reabsorption of glucose by the kidney or the absorption of glucose by the gastrointestinal tract. Thus, *insulin is not a general "catalyst" of carbohydrate metabolism*, as sometimes supposed, but instead it shows a considerable degree of specificity as to the type of cell or organ which it affects. The muscles, because of their large mass *in toto*, must then be a major site of action of the hormone.

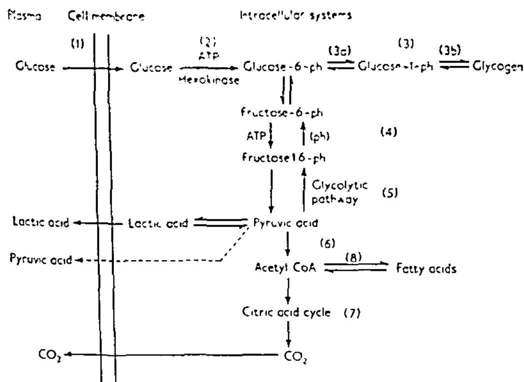


Fig. 1. Outline of carbohydrate metabolism in muscle: possible sites of action of insulin.

2. *Mechanism of Action of Insulin*

The precise locus of action of insulin in biochemical or physiological terms has not yet been fully elucidated. Stadie (1954), Levine and Goldstein (1955), and Ross (1956) have reviewed the theories which have been held and the evidence available on the mechanism of insulin action. The scheme in Fig. 1 indicates the principal points of attack which have been considered.

There has been no convincing evidence presented to indicate that insulin, or its lack, can affect intermediate stages of either the anaerobic metabolism of carbohydrate [Fig. 1, (5)] or the aerobic oxidation of the

as glycogen in skeletal muscle, and that the rest of the glucose may have been oxidized. Apart from the fall in blood glucose induced by insulin, the deposition of glycogen in skeletal muscle is now perhaps the best attested effect of the hormone; for it has been observed in nearly every case in which convulsive effects of hypoglycemia have been avoided and in which the supply of carbohydrate has been sufficient to allow an increase in glycogen to be seen.

In 1940–1941, Gemmill and Gemmill and Hausman (1941) reported that when segments of rat diaphragm were incubated *in vitro*, added insulin increased the rates of uptake of glucose from the medium, of deposition of glycogen in the diaphragm, and of disappearance of total carbohydrate from the system. Since Gemmill's original observations, the isolated rat diaphragm has been used in most of the studies which have been undertaken on the mode of action of insulin in muscle [reviewed by Krahl (1951) and by Stadie (1954)]. Similar effects have been observed in strips of other types of skeletal muscle, and also in the isolated perfused heart (Bleehen and Fisher, 1954). In all of these, the amounts of insulin required to elicit significant effects are near the physiological range, and the effects are evident within a short time, paralleling the time course of the hypoglycemia which develops after insulin is given to the intact animal. The evidence thus indicates that insulin acts upon the metabolism of carbohydrate in skeletal muscle and heart without the intervention of any other organs in the body.

Although there has been no question that skeletal and cardiac muscles constitute one site of action of insulin, there has been controversy as to the relative participation of the muscles as compared with other organs or systems in this respect. It now appears that another site of action is adipose tissue, in which also it is possible to demonstrate activity of insulin *in vitro*. Other tissues and organs, including smooth muscle, quite possibly also may behave in response to insulin as do skeletal and cardiac muscle and adipose tissue, but definitive studies are available for only a few other systems. The liver, often considered to be a primary site of action of the hormone, in fact seems generally to be much more responsive to the hypoglycemic action of insulin, releasing glucose to the blood, than it is to any immediate augmentative effect which the insulin may have on the uptake of glucose or on its metabolism (Cori *et al.*, 1930; Bridge, 1938; Wall *et al.*, 1957); and it has not been possible to demonstrate immediate effects of insulin in the liver *in vitro* similar to those seen in muscle. The brain and the red

the operative materials in the extract of diabetic muscle), and that this inhibition was relieved by insulin.

A singular feature of this work was the very great irregularity of the results: in only about one-half of the attempts made could an insulin effect (interpreted always as relief of inhibition) be seen. The presumed "diabetogenic" factor from the anterior pituitary was extraordinarily labile, and neither the pituitary nor the adrenocortical factor could be identified with any known hormone from these glands. Other investigators attempting to repeat this work (reviewed by Stadie, 1954; Ross, 1956) have reported either a much smaller proportion of successful trials or no significant effect on the hexokinase reaction either of insulin or of the presumed contrainsulin factor in diabetic muscle. In addition, nonspecific or artifactual augmentation and inhibition of this reaction have been reported frequently (Stadie *et al.*, 1950; Stadie, 1954; Long and Thompson, 1955). In sum, these investigations do not offer any conclusive evidence for the hexokinase reaction as the site of insulin action in muscle.

The concept that insulin might act by promoting the penetration of glucose through the cell membrane, rather than by directing the fate of intracellular glucose, has been proposed often in the past; but until recently, little direct evidence had been presented either for or against this view. Serious consideration of this idea was no doubt delayed by the general impression, current for many years, that the process of glucose uptake into the cell was synonymous with or included formation of the "active" hexose phosphates. Also, it was widely supposed, but with little supporting evidence, that the action of insulin was specific for glucose. Recently, however, several sets of observations have forced the rejection of the latter ideas and have indicated that insulin may have a profound effect upon the transport from the extracellular fluid into the muscle cell not only of glucose but also of several other sugars.

In work beginning in 1949, Levine and co-workers were the first to show the latter phenomenon conclusively (Levine *et al.*, 1950; Goldstein *et al.*, 1953a; Levine and Goldstein, 1955). In eviscerated nephrectomized animals, they found that insulin would greatly increase the apparent volume of distribution of galactose (which is utilized only very slowly in this preparation) and of D-xylose and L-arabinose (pentoses which are utilizable scarcely at all in mammalian tissues). Since these responses could not be attributed to an action of insulin on the hexokinase reaction, it was concluded that insulin must act on the

2-carbon intermediates of carbohydrate and fat metabolism via the citric acid cycle (7). There have been some suggestions that the oxidation of pyruvate (6–7) might be susceptible to augmentation by insulin; but either the observations in these cases have not been confirmed, or they have been found to be confined to quite special circumstances (as the partial maintenance by insulin of the oxygen uptake of pigeon breast muscle mince incubated with pyruvate). The latter observation may well have other explanations. Although increased glycogen deposition (3), increased oxidation of glucose (7), and increased fat formation (8) have all been demonstrated in animals given insulin, the relative proportions of material undergoing these pathways in any one organ have not been shown to be altered materially. There is thus little to suggest a differential action of insulin on the direction of the metabolic fates open to the hexose phosphates in muscle. Since insulin certainly does augment the deposition of glycogen in this tissue, but seems not to affect later stages of carbohydrate metabolism directly, attention has been directed in recent years to the earlier stages of the assimilation of glucose: the penetration or transport of the sugar into the cell (1), and the first demonstrable intracellular reaction, the "activation" of the glucose by phosphorylation (2). These two stages, earlier thought to be integral, have now been shown quite convincingly by Park and others to be separable processes (see below).

The idea that insulin acts by augmenting the rate of phosphorylation of glucose by the hexokinase reaction was brought to the fore by the work of the Coris (Cori, 1946; Colowick *et al.*, 1947) and has received wide currency since that time. Unfortunately, the frequent incidental references in the literature to this work have been for the most part inaccurate. In fact, the data presented by these workers were not taken to show any direct action of insulin on this system. Rather, it was reported that in crude cell-free extracts prepared from the muscles of diabetic rats, and containing added adrenocortical extract, insulin would diminish the usual initial lag in the rate of the hexokinase reaction. The result of the addition of insulin was then to increase the final uptake of glucose and phosphate in the system, although the actual rate of the reaction in most of its course appeared to be unchanged. No such action was seen in extracts from normal muscle. From further similar studies on a crude hexokinase preparation from brain, it was concluded that this reaction could be inhibited for a short time by the combined action of anterior pituitary and adrenocortical substances (presumably

Thus, recent evidence strongly supports the conclusion that insulin augments some aspect of the mechanism whereby glucose and other sugars gain access to the interior of the muscle cells. The more intimate nature of this system is unknown.

3. *Diabetes and Exercise*

Although the depancreatized or alloxan diabetic animal lacks insulin, the muscles maintain normal concentrations of glycogen in the resting postabsorptive state. Glycogenolysis is normal after stimulation, but resynthesis during recovery has been reported to be relatively slow (Lukens, 1934). Also, deposition of glycogen after a glucose meal may be less than normal, for the glycogen of the diaphragm, which ordinarily increases severalfold in this circumstance, did not change in the fed diabetic rat (Illingworth and Russell, 1951).

Insulin is not the only factor which can augment the uptake of glucose by the muscle cell, and it is not an absolute requirement for utilization of carbohydrate by the diabetic under all circumstances. Clinicians have recognized for some time that less insulin is needed by the diabetic during heavy exercise, and Ingle and his coworkers (1950a, 1951; Ingle and Nezamis, 1948a) have shown clearly that exercising muscle can remove glucose from the blood rapidly in the complete absence of insulin.

That muscular work, like insulin, increases the penetration of certain nonutilizable sugars into muscle has now been demonstrated by several investigators (Goldstein *et al.*, 1953b; Huyck and Kruhoffer, 1953; Sacks and Smith, 1958). The specificity of exercise in respect to structure of the sugars affected was quite similar to that of insulin, described above. According to Sacks and Smith, the effect of exercise on the penetration of L-arabinose was related roughly to the rate of work, and the effects of insulin and work appeared to be additive. In exercising muscle in diabetic rats, the work capacity was normal, and the addition of considerable amounts of insulin and glucose did not enable the muscles to do more work (Ingle *et al.*, 1951). It is not known whether insulin and exercise affect the same mechanism for glucose uptake.

The fate of the glucose taken up during exercise has not been studied in detail, but presumably it is used in normal fashion. The ability of the muscles to take up glucose during exercise may account for the maintenance of normal glycogen stores in the absence of insulin.

The cardiac glycogen of the diabetic animal is commonly above

"transport" of these sugars, and by inference, on the "transport" of glucose as well.

Further observations on the volume of distribution of a variety of other sugars and closely related substances in the presence or absence of insulin led Levine *et al.* to suggest that the action of insulin was specific for monosaccharides having the same configuration on the first three carbon atoms as D-glucose. In the resting eviscerated animal without insulin, the volume of distribution was much the same for all of the nonutilizable substances studied, indicating only limited intracellular penetration by any of the sugars under these conditions. Although more recent evidence indicates that this may be too simple a view of the specificity of insulin action, the general thesis has been upheld. The action of insulin on the penetration of galactose and certain other sugars into muscle cells has been amply confirmed by analysis of tissue as well as by measurement of distribution and uptake (Wick and Drury, 1953; Haft *et al.*, 1953; Huycke and Kruhøffer, 1953; Park and Johnson, 1955; Park *et al.*, 1957; Nakada and Wick, 1956; Fisher and Lindsay, 1956; Sacks and Bakshy, 1957; Sacks and Smith, 1958; Helmreich and Cori, 1957; Kipnis and Cori, 1957; Resnick and Hechter, 1957; Landau *et al.*, 1958).

Direct evidence that glucose enters resting muscle relatively slowly without insulin, but, like the nonmetabolizable sugars, is taken up into the cell as free glucose when insulin is present, has now been presented. Park *et al.* (1955) have shown that in isolated diaphragm the rate of the hexokinase reaction ordinarily is limited mainly by the rate of access of glucose, and little or no free glucose is found within the cells. However, when the hexokinase reaction itself becomes limiting on glucose utilization (either at low temperatures or when the extracellular concentration of glucose is extremely high), some free sugar is now present, and in these circumstances insulin is readily shown to augment the accumulation of glucose inside the cell.

In the intact or eviscerated animal, insulin has been shown also to enable some intracellular accumulation of free glucose as well as of other sugars in heart, diaphragm, and gastrocnemius muscle (Park and Johnson, 1955; Park *et al.*, 1957). Fisher and Lindsay (1956), using the isolated perfused rat heart, have demonstrated clearly an increment of free sugar within the cells in presence of insulin. Some degree of competition between glucose and galactose for the "transport" mechanism has also been seen (Wick and Drury, 1953; Fisher and Lindsay, 1956).

in normal tissues, but the authors did not agree as to the effects of glucose in the medium and of other conditions on this phenomenon. More complete and systematic studies of the effect of insulin on the uptake of amino acids into isolated tissues are needed before these results can be interpreted.

Muscle work, which increases the uptake of glucose into the tissues, had no effect on the release of amino acids into the blood (Ingle *et al.*, 1955).

B. Glucagon

The hyperglycemic factor obtained from the pancreas and thought to arise in the α cells, called glucagon, is glycogenolytic in liver but has not been shown to affect the metabolism of muscle to any notable degree (reviewed by de Duve, 1953). Glucagon is said not to cause an increase in blood lactate; and when it was given with insulin so as to maintain normal blood glucose levels in intact rabbits, no changes in muscle glycogen were seen. This is in contrast to the action of adrenaline, which in the same circumstances is strongly glycogenolytic in muscle. Several investigators agree that glucagon has no effect on the arteriovenous difference in glucose concentration across a limb except as would be expected from the hyperglycemia alone (Tomizawa and Hyde, 1958). Ingle *et al.* (1953) did not see any hyperglycemic action of this material in eviscerated rats given glucose with or without insulin. In eviscerated rabbits, Drury *et al.* (1954) reported a small reduction in the rate of glucose disappearance when glucagon was given with "maximal" insulin dosage but not with "low" insulin supply. No change in the production of $C^{14}O_2$ from labeled glucose was seen. In the isolated diaphragm *in vitro*, glucagon has been reported to inhibit partly the usual effect of insulin on glucose uptake (R-Candela, 1953; Snedecor *et al.*, 1955). In the absence of insulin, no effect of glucagon could be demonstrated in this system. The amounts of glucagon used were very large compared to the amounts of insulin present, so this may represent a nonspecific effect.

C. Adrenomedullary Hormones

The varied effects of adrenaline and of noradrenaline on muscle are conveniently classified as metabolic or functional, the latter expressed generally as alterations in the contractility or excitability of the tissue. The metabolic effects have been observed mainly in skeletal muscle,

normal and may be very high (e.g. Evans and Bowie, 1936; Lackey *et al.*, 1944; Illingworth and Russell, 1951). The reason for this condition is not known. It might be associated with the continuous work of the heart muscle in the presence of hyperglycemia; or it could be related to a relative excess of pituitary growth hormone, which is known to augment the cardiac glycogen.

4. *Effect of Insulin on Nitrogen Metabolism in Muscle*

Insulin is necessary not only for normal growth and development of the body and for the action of growth hormone on nitrogen balance and growth, as mentioned earlier in this chapter, but it is required also for nitrogen balance in the adult (Atchley *et al.*, 1933; Chaikoff and Forker, 1950). Possible mechanisms of action of insulin on nitrogen metabolism have been reviewed by Munro (1956). That muscle is probably the site of at least part of the action of insulin on nitrogen balance is indicated by the facts that in the eviscerated animal amino nitrogen is released constantly into the blood, and that this loss can be suppressed wholly or in part by the administration of insulin (Mirsky, 1938; Frame and Russell, 1946; Ingle *et al.*, 1947; Russell and Cappiello, 1949; Bollman *et al.*, 1953). This effect is independent of the blood sugar level or of the amounts of carbohydrate given. Diminution in the rate of loss of amino acids can be brought about by doses of insulin near the physiological range (0.1–1.0 units per kilogram per hour), and with large doses even some reduction in the plasma amino nitrogen level has been seen (Ingle *et al.*, 1956). When extra mixed amino acids were given to the eviscerated rat, quite small amounts of insulin (0.05 units per kilogram per hour) appeared to promote the disappearance of the administered amino acids (Russell, 1953b). These results suggest that insulin not only prevents the loss of amino acids from the peripheral tissues but may actually enhance the uptake of amino acids.

A few attempts have been made to demonstrate more directly an action of insulin on the incorporation of amino acids directly into muscle. Forker *et al.* (1951) observed that the uptake of S^{35}-methionine into the proteins of leg muscle in the eviscerated diabetic dog was much less than in the muscle of control animals. Small amounts of insulin given to the diabetic dog at the time had no effect, but pretreatment for 3 days restored the uptake. Sinex *et al.* (1952) and Krahl (1953) have reported on the uptake of certain C^{14}-labeled amino acids into the proteins of diaphragm *in vitro*: some augmentation by insulin was seen

and may be depleted by half within an hour, and the blood lactate is doubled or trebled, reaching a peak in about 45 min. to 1 hour. The blood lactate then declines, but the muscle glycogen remains depleted for many hours. Lactate and hexose monophosphates (mainly glucose-6-phosphate) in muscle increase also, the latter remaining elevated for several hours (Cori and Cori, 1931).

All types of skeletal muscle are believed to share in the glycogenolytic response to adrenaline, although comparative data are available for only a few muscles (in the rat, gastrocnemius, rectus femoris, diaphragm, and abdominal). Cardiac glycogen, however, differs notably from skeletal muscle in its sensitivity to adrenaline. Loss of glycogen may be induced by heavy dosage, but if so, it is evident only for a brief period; and it is not seen after moderate dosage, even that which induces extensive glycogenolysis in skeletal muscle in the same animal (reviewed by Bloom and Russell, 1955; Ellis, 1956). In some conditions, an increase in cardiac glycogen may be seen. Smooth muscle appears to respond with some degree of glycogenolysis. The uterus (including both myometrium and endometrium) was partly depleted of glycogen by a rather large dose of adrenaline given intraperitoneally but not if given subcutaneously (Kostyo and Leonard, 1955). In several types of smooth muscle *in vitro* (rabbit intestine, bovine trachea and coronary vessel, guinea pig uterus), glycogenolysis and increased lactate production have been observed in presence of adrenaline (Mohme-Lundholm, 1953, 1957: Lundholm and Mohme-Lundholm, 1957). In general, the degree of glycogenolysis induced by adrenaline was correlated with the relaxant or inhibitory action of the hormone in the particular preparation.

The glycogenolytic action of adrenaline is readily observed in frog leg muscle or in rat diaphragm *in vitro* (Hegnauer and Cori, 1934; Riesser, 1947; Walaas and Walaas, 1950; Walaas, 1955 p. 126; Stadie *et al.*, 1951). Relative depletion of glycogen (or diminished deposition of glycogen) occurs in presence or absence of glucose or of insulin in the medium. Of the glucose taken up, a much larger fraction appears as lactate.

b. Noradrenaline. Unlike adrenaline, noradrenaline appears to have very little glycogenolytic effect in skeletal muscle. Although it does exhibit some degree of hyperglycemic action (in potency less than one-tenth that of adrenaline), it has not been seen to cause any diminution

the functional changes mainly in heart and smooth muscle; but this division is not exclusive. The little that is known of relationships between metabolic and functional activities is indicated below. Mechanisms of action of these hormones in respect to function are considered in detail elsewhere in this volume in connection with the effects of sympathomimetic drugs on skeletal and smooth muscle and on the heart (Volume III, chapters I and II). Accordingly, this section is devoted mainly to metabolic aspects, with a brief outline of the physiological rôles of these hormones as they affect functional activity in various muscles of the body.

1. Carbohydrate Metabolism and Related Processes

The confirmed metabolic action of these hormones in muscle is concerned mainly with certain aspects of the metabolism of carbohydrates. The calorigenic effect of adrenaline, and effects on the movements of certain ions into and out of muscle, may be related to changes in carbohydrate metabolism. Some alterations in the metabolism of fat and of nitrogenous substances have been indicated also to attend the administration of the adrenalines in the intact animal, but these effects do not appear to be important in muscle. The metabolic effects of sympathomimetic amines, including those in muscle, have been reviewed extensively by Ellis (1956).

a. Actions of Adrenaline on Carbohydrate Metabolism in Muscle. The classical effects of adrenaline on the blood glucose and liver glycogen were shown by the work of the Coris to be only partly accounted for by its glycogenolytic action in liver (for review, see Cori, 1931). Equally or often more marked glycogenolysis was shown to occur in skeletal muscle. In this tissue, however, glucose is not liberated as it is in liver because muscle lacks the necessary glucose-6-phosphatase; but instead lactic acid is formed and released in quantity. The blood lactate is then taken up by the liver and reconverted to glycogen and glucose, thus contributing indirectly but substantially to the prolonged hyperglycemia seen after adrenaline.

The amounts of adrenaline required to elicit this effect on muscle are of the same order as those which induce hyperglycemia, and may be less than those which increase the blood pressure. With moderate dosage (e.g. 0.2 mg. per kilogram subcutaneous), the glycogen content of a muscle such as the gastrocnemius in the rat is diminished within 15 min.

and may be depleted by half within an hour, and the blood lactate is doubled or trebled, reaching a peak in about 45 min. to 1 hour. The blood lactate then declines, but the muscle glycogen remains depleted for many hours. Lactate and hexose monophosphates (mainly glucose-6-phosphate) in muscle increase also, the latter remaining elevated for several hours (Cori and Cori, 1931).

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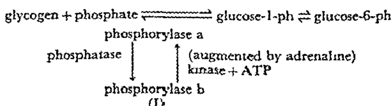
only a few muscles (in the rat, gastrocnemius, rectus femoris, diaphragm, and abdominal). Cardiac glycogen, however, differs notably from skeletal muscle in its sensitivity to adrenaline. Loss of glycogen may be induced by heavy dosage, but if so, it is evident only for a brief period; and it is not seen after moderate dosage, even that which induces extensive glycogenolysis in skeletal muscle in the same animal (reviewed by Bloom and Russell, 1955; Ellis, 1956). In some conditions, an increase in cardiac glycogen may be seen. Smooth muscle appears to respond with some degree of glycogenolysis. The uterus (including both myometrium and endometrium) was partly depleted of glycogen by a rather large dose of adrenaline given intraperitoneally but not if given subcutaneously (Kostyo and Leonard, 1955). In several types of smooth muscle *in vitro* (rabbit intestine, bovine trachea and coronary vessel, guinea pig uterus), glycogenolysis and increased lactate production have been observed in presence of adrenaline (Mohme-Lundholm, 1953, 1957: Lundholm and Mohme-Lundholm, 1957). In general, the degree of glycogenolysis induced by adrenaline was correlated with the relaxant or inhibitory action of the hormone in the particular preparation.

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b. Noradrenaline. Unlike adrenaline, noradrenaline appears to have very little glycogenolytic effect in skeletal muscle. Although it does exhibit some degree of hyperglycemic action (in potency less than one-tenth that of adrenaline), it has not been seen to cause any diminution

in muscle glycogen, nor in moderate dosage does it have any effect on the blood lactate (Lundholm, 1950; Bearn *et al.*, 1951; Bloom and Russell, 1955). With very large dosage, the blood lactate may rise later; but this may well be the result of anoxia due to vasoconstriction or other toxic effect of the near-lethal amounts of hormone used. In heart, either no effect or an increase in glycogen has been seen (Bloom and Russell, 1955). In smooth muscle *in vitro*, glycogenolysis occurred in presence of noradrenaline, but only at higher concentrations than required of adrenaline (Mohme-Lundholm, 1956). This action was again correlated with the inhibitory activity of the amine.

c. Mechanism of the Glycogenolytic Effect. As shown many years ago by the Coris, the fundamental action of adrenaline in muscle is quite similar to that in liver. Since hexose monophosphates accumulate when adrenaline is given, the action of the hormone must lie in some phase of glycogenolysis [Fig. 1 (3)], rather than in glycolysis proper. The extensive investigation of the mechanism of action of adrenaline and of glucagon in liver by Sutherland and Cori (1951) has shown that the phosphorylase reaction [Fig. 1, (3b)] is limiting during glycogenolysis, and that its rate is enhanced in both directions by both of these substances. The phosphorylase of muscle had been shown earlier by the Coris and their colleagues to occur partly in a form which was inactive in the absence of added adenylic acid (called phosphorylase b) and partly in an active form not requiring this substance (phosphorylase a). More recent studies by Sutherland and co-workers (Wosilait and Sutherland, 1956; Rall *et al.*, 1956a, b; Krebs and Fischer, 1956) have shown that in both liver and muscle the enzyme phosphorylase can be inactivated and reactivated by specific enzyme systems within the respective organs; in both cases, phosphate is split off the phosphorylase by the inactivating enzyme, which is therefore a type of phosphatase, and is replaced by transfer of phosphate from ATP by an enzyme which is therefore called a dephosphophosphorylase kinase. These relationships may be indicated as shown in scheme (I).



Adrenaline has been shown to accelerate the reactivation process and the concomitant activity of the kinase in purified liver preparations (Rall *et al.*, 1956a, 1957). A similar type of activity appears to occur in muscle, for the phosphorylase in isolated diaphragm was reactivated by adrenaline *in vitro* (Sutherland, 1951). Further, in either the muscles of the intact rat or in isolated frog muscle, adrenaline increased the proportion of phosphorylase found in the active form (Cori and Illingworth, 1956; Leonard, 1957). Noradrenaline was much less active than adrenaline in this respect.

In muscle stimulated to fatigue, the phosphorylase is largely in the inactive form; but as demonstrated by Cori and Illingworth, adrenaline diminishes the rate of inactivation during stimulation and accelerates the rate of resynthesis of the enzyme during recovery. Since glycogenolysis at very rapid rates may be needed during muscular activity, particularly in relative anoxia, it seems likely that a high concentration of active phosphorylase would be required to support continued work at the expense of glycogen under these conditions. It is reasonable to suppose, therefore, that the reported effect of adrenaline in delaying fatigue of muscle may be in part at least a consequence of its action on the phosphorylase activating system.

An unexplained phenomenon in connection with the action of adrenaline on the phosphorolysis of glycogen is that although phosphorylase catalyzes the reaction in both directions and the position of equilibrium of the reaction is on the side of glycogen, the hormone always, without exception, in skeletal muscle, accelerates the breakdown of glycogen. One can see that glycogenolysis might be expected in any case where the first product of the reaction, glucose-1-phosphate, was being continually removed via glucose-6-phosphate and the reactions of the latter ester. However, the increase in hexose monophosphate which occurs regularly in muscle during the action of adrenaline seems to indicate that the rate of removal of the esters does not in any case keep pace with their formation from glycogen. Moreover, when glucose is being taken up and glycogen is normally being formed at a rapid rate, as in the presence of high concentrations of glucose or of insulin, one would expect that increasing the concentration of active phosphorylase by the action of adrenaline would tend to augment the rate of the reverse reaction, and hence to increase the formation of glycogen or at least diminish its breakdown. In fact, in such circumstances the glycogenolytic effect of adrenaline remains uninhibited both *in vivo* and *in vitro*

(Cori and Cori, 1929; Hegnauer and Cori, 1934; Riesser, 1947; Walaas, 1955 pp. 109, 126). It is probable that the present concept of the action of adrenaline may represent an oversimplification of its mechanism in the intact cell.

d. Effects of Adrenalines on Utilization of Carbohydrate. The question whether the hyperglycemia which occurs after adrenaline may be due in part to reduction in the uptake and utilization of carbohydrate by muscle and other tissues, as well as in part to increased glycogenolysis, has been debated for the past thirty years. The evidence on this point has been well summarized by Ellis (1956). In general, no effect of adrenaline on the peripheral arteriovenous difference or on glucose assimilation has been seen at normal blood glucose levels in the absence of insulin. However, at elevated blood sugar levels the arteriovenous difference is less after adrenaline than would be expected at the same blood glucose concentrations induced by administration of glucose; and if adrenaline is given with glucose to an intact subject, or with glucose and insulin to an eviscerated animal, the rate of removal of the glucose from the blood may be substantially reduced (Ingle and Nezamis, 1950; Fritz *et al.*, 1957). This suggests that adrenaline may regularly depress the assimilation of glucose by peripheral tissues, presumably including muscle, whenever the basal rate is sufficiently rapid to allow this action to be seen. This expectation appears to have been confirmed by observations on the rat diaphragm *in vitro*, in which adrenaline in glycogenolytic concentration has diminished the uptake of glucose from the medium (Walaas and Walaas, 1950; Stadie *et al.*, 1951; Walaas, 1955, p. 109). The uptake of mannose and of fructose has been reported also to be inhibited (Walaas, 1955, p. 109). It has been suggested that this effect may be due to inhibition of the hexokinase system by glucose-6-phosphate, which accumulates as the result of adrenaline action and which is known to be a potent inhibitor of the enzyme. The arguments in this connection, which remain unsettled, have been reviewed by Ellis (1956). The actions of adrenaline and of insulin on glucose uptake *in vitro* and on glucose assimilation *in vivo* appear to be to some extent mutually antagonistic; but adrenaline did not interfere with the action of insulin on the volume of distribution of galactose in the eviscerated dog (Fritz *et al.*, 1957) It seems unlikely, therefore, that adrenaline and insulin affect the same mechanism for glucose assimilation in muscle.

e. Oxygen consumption. As is well known, adrenaline given to the intact subject brings about a substantial increase in oxygen consumption. The mechanism of this effect is not altogether clear (Griffith, 1951; Ellis, 1956). Lundholm (1949) has found that the infusion of lactic acid, so as to reproduce the lactacidemia seen after adrenaline, produces the same degree of hypermetabolism as does the hormone. Since lactate is known to increase the metabolic rate of many tissues *in vitro*, Lundholm suggests that the increase in oxygen uptake is due to the metabolism of the lactic acid liberated by glycogenolysis in muscle. There is not, however, any strong evidence that adrenaline increases the metabolic rate of the muscle itself other than by increasing its temperature or work output. When the latter factors have been kept constant, usually no change in oxygen uptake has been attributable to direct hormonal effects on the metabolism of peripheral tissues or heart either *in vivo* or *in vitro*. The calorigenic response to adrenaline may then be confined largely to the viscera and liver (Bearn *et al.*, 1951).

f. Inorganic Ions in Muscle. As has long been known, adrenaline depresses the serum inorganic phosphate. This effect probably may be associated with the phosphorolysis of glycogen and the accumulation of hexose phosphate which occurs in muscle. Inorganic phosphate in the muscle does not change or may fall slightly in these conditions (Cori, 1931; Cori and Cori, 1931). Ellis (1956) considers that the change in serum phosphate also may be due in part to the action of insulin secreted reflexly in response to hyperglycemia after adrenaline. However, there is no evidence that insulin affects the hexose phosphate content of muscle.

The administration of adrenaline is commonly followed by a brief transient increase in serum potassium and then a longer depression (Ellis, 1956). The initial hyperkalemia is due to release of potassium from the liver, but the hypokalemia probably is related in part to the effects of the hormone in muscle. A diminution in the rate of loss of potassium from muscle *in vitro* in presence of adrenaline was correlated in time with the duration of action of the hormone on muscle response, and then later the rate of loss of potassium was increased (Goffart and Perry, 1951). These effects are in accord with what is known of movements of potassium into and out of muscle when adrenaline is given *in vivo* (Ellis, 1956). It is possible that the potassium accompanies phosphate taken up to form the hexose phosphates, and then leaves the cell

later, as the hydrogen ions produced in phosphorolysis and glycolysis are neutralized by nondiffusible material within the cell.

2. *Functional Effects of Adrenomedullary Hormones in Muscle*

a. Smooth Muscle and Heart. The classic effects of adrenaline and of other sympathomimetic amines include excitation or contraction of many smooth muscles and inhibition or relaxation of others. In general, they induce constriction of arterioles and precapillary "sphincters" in most areas of the body (with a few notable exceptions, indicated below), and contraction of some intestinal sphincters, the splenic capsule, the dilator pupillae and nictitating membrane, the pilomotor muscles, and the retractor penis. On the other hand, the bronchial musculature and most of the visceral muscles, except certain sphincters, are inhibited or relaxed. The uterus usually is inhibited but it may be excited, depending on the species and on the physiological state of the organ. In the heart, as is well known, the adrenalines increase the force, frequency, and amplitude of contraction; these effects are discussed in detail elsewhere in this treatise (Volume III, Chapter II). The coronary vessels are relaxed and circulation through the heart muscle is increased by these agents.

The two hormones, adrenaline and noradrenaline, have for the most part similar effects qualitatively, but they may differ considerably in quantitative potency in different organs. The impression is often given that norepinephrine is the more active as an excitor and that it does not have inhibitory actions; but these generalizations are inaccurate. In fact, in the majority of excitatory effects, including those on the heart, the two substances are of roughly equal potency or else adrenaline is somewhat more active. Noradrenaline has appreciable inhibitory effects, although in most cases it is less active than adrenaline. As pointed out by Gaddum and Holtzbauer (1957), the potency ratio for a given organ may vary widely, the value often depending on the particular experimental conditions.

The comparative actions of the hormones on the peripheral vasculature, on the other hand, present a special point of difference: whereas noradrenaline is constrictor in nearly all areas (except the coronary), and adrenaline too is constrictor in skin, viscera, and elsewhere, adrenaline has little or no constrictor action on the blood vessels of skeletal muscle, and it often behaves as an active dilator agent in this tissue (Barcroft and Konzett, 1949; Bock *et al.*, 1955). In consequence, nor-

adrenaline greatly increases the peripheral vascular resistance and so increases diastolic as well as systolic pressure; but adrenaline has much less or no effect on resistance and hence its effect on the diastolic and mean blood pressure is considerably smaller than is that of noradrenaline. Since the effects of the two substances on the heart are similar, the result of this difference in action on a major group of blood vessels is that noradrenaline is a more potent pressor agent than adrenaline. It is probably this difference in pressor activity which has led to the erroneous impression, mentioned above, that noradrenaline is the more active excitatory agent.

The reason for this difference in the response of the blood vessels in skeletal muscle to the adrenalines is not certain. One hypothesis is concerned with the metabolic actions of the hormones in muscle. As mentioned earlier, Mohme-Lundholm has observed that the glycogenolysis and lactic acid production induced by these substances may be correlated with their inhibitory effects on several types of smooth muscle. It has long been known that reduction in the pH inhibits contractions of smooth muscle *in vitro* and that it has a vasodilator effect *in vivo*. It seems likely, therefore, that the relaxing effect of adrenaline in the arterioles of skeletal muscle is the result of the formation and release of acid during glycolysis in the muscle cell, and Lundholm (1956) has presented evidence of close parallelism between glycogenolytic and vascular effects *in vivo*. Since noradrenaline has little effect on glycolysis in skeletal muscle, the difference in response of the blood vessels of muscle to these substances may well be explicable on this basis. It is possible, too, as Mohme-Lundholm suggests, that the lesser inhibitory action of noradrenaline in other types of smooth muscle is related to its relative ineffectiveness as a glycogenolytic agent. To what extent other variations in the metabolic responses to these hormones can account for their varying activities in different organs is not yet known.

b. Skeletal Muscle. Although the medullary hormones appear to have little or no direct excitatory or inhibitory activity in skeletal muscle, such as may be elicited easily in heart and smooth muscle, adrenaline has been shown frequently to affect the contractile response of voluntary muscle to stimulation under certain conditions. In their pioneer work on adrenal extracts, Oliver and Schäfer in 1895 first showed that the response to stimulation in skeletal muscle was prolonged in animals injected with the extracts, and Gruber, in 1914, observed that adrena-

line increased the ability of fatigued muscle to respond to continued stimulation. The latter phenomenon has been much studied in connection with the role of sympathetic activity in muscular contraction. Until recent years, this effect has been attributed usually to some action of adrenaline on neuromuscular transmission (reviewed by Burn, 1945). However, Brown *et al.* (1948), in careful studies of isolated innervated diaphragm, could find no evidence that this was so. In partially fatigued muscle stimulated either directly or indirectly, the tension developed in response to a single shock or in continued tetany was increased markedly when adrenaline was added. The maximum effects were seen at 2 to 5 min. after introduction of the hormone and were not related to changes in the action potentials. From somewhat similar studies in working muscle, Goffart and Ritchie (1952) suggested that the action of adrenaline was to prolong the active state. Montagu (1955), observing that the action of adrenaline on the response to indirect stimulation was altered in media of varying ionic composition, concluded that transmission was affected as well as the maintenance of the active state, but that the former effect might be related to movements of potassium and calcium ions consequent to acid production in the muscle under the influence of adrenaline. These observations all suggest that the primary site of action of adrenaline in the responses of fatigued or working muscle lies in the muscle cell itself.

Another phenomenon, which may possibly be related to the action of adrenaline on this contractile response of muscle, has been reviewed by Burn (1945). In the denervated muscle, acetylcholine induces contractions, and this effect is potentiated by adrenaline. This suggests, of course, an action of adrenaline on some phase of transmission. However, as Burn points out, adrenaline also increases the contractile response of denervated muscle to the tetramethylammonium ion; it potentiates the action of prostigmine in working muscle but antagonizes its inhibitory action in fatigued muscle; and it has little effect on the action of eserine. No common explanation seems possible for all of these actions of adrenaline in terms of transmission alone. It may be that the fundamental site of action of the hormone is within the muscle cell in these cases also.

Little information is available on the relative activity of noradrenoline in these respects in skeletal muscle.

The mechanism of the action of adrenaline in the stimulated skeletal muscle has no certain explanation and may be complex. However, as

mentioned above (p. 023), adrenaline has been found to inhibit the inactivation of phosphorylase which otherwise occurs during stimulation and to hasten greatly the reactivation of the enzyme during recovery (Cori and Illingworth, 1956). Acetylcholine and some other stimulants decreased the amount of active phosphorylase. It seems possible that some part of the phenomena just described could well be related to the facilitation of glycogenolysis by adrenaline during activity in muscle. Obviously, more detailed examination of this hypothesis is required.

3. *Adrenodemedullation*

If after removal of the adrenal medullae, the adrenal cortices are allowed to regenerate fully, the animal appears to be perfectly normal in most respects; it is normally active and is not subject to the myasthenia seen in adrenocortical deficiency. There seem to have been few detailed studies on the composition or function of muscle in this condition, nor on the responses of the muscle of such animals to the adrenalines. The storage and metabolism of carbohydrate in resting muscle are little affected, and only slightly higher glycogen levels have been seen in certain muscles in the fasting rat (Bowman, 1956). However, glycogenolysis does not occur in skeletal muscle when the animal is subjected to hypoglycemia or to operative trauma (Cori and Cori, 1931; Russell, 1938b, 1942; Bloom and Russell, 1955). This indicates that the adrenal medullae constitute the only source of significant quantities of circulating adrenaline after general sympathetic stimulation. Since noradrenaline is not perceptibly glycogenolytic in muscle, the secretion of this amine elsewhere in the body would not be expected to affect skeletal muscle.

D. Adrenocortical Hormones

One of the symptoms of untreated adrenocortical deficiency most frequently noted is weakness and easy fatigability of the muscles. This "myasthenia" can be prevented or overcome quite readily by suitable treatment with any of the several active cortical steroids. However, the mechanisms concerned are by no means clear. In view of the manifold and diverse activities displayed by the adrenal steroids, it is probable that their actions in this respect are complex. Further, it may be noted that little or no convincing evidence has been adduced for positive effects of the cortical hormones on muscle function in intact normal

subjects. As is true with respect to many other aspects of cortical hormone activity, it is likely that the hormone does not itself limit muscle function; instead, it may play mainly a permissive rôle, in that it is required for or allows normal activity of other mechanisms.

For convenience, the effects of adrenal deficiency and of cortical hormones may be classified as those related to electrolytes and water, to metabolism of carbohydrate and protein, and to functional responses. Of the latter, only a few are explicable at present in terms of changes in either ionic composition or metabolism.

1. *Electrolytes and Water*

Effects of adrenal deficiency and replacement on the ionic composition of muscle are related for the most part to alterations in the electrolyte pattern of the plasma and extracellular fluid. These are largely the result of effects of the hormone on renal function, but there are indications that some extrarenal effects also may occur, particularly in respect to potassium and to water.

a. Plasma Electrolytes in Relation to Muscle. The classic picture of adrenal deficiency includes abnormal losses by excretion (mainly in the urine, but also in saliva, sweat, and gut) of sodium, chloride, and water, and diminished renal excretion of potassium. The result is that the concentrations of sodium and chloride ions in the plasma fall and that of potassium rises. With the fall in osmotic concentration in the extracellular fluid, water then tends to move into the cells, and the extracellular fluid volume is reduced, often to critical levels. Concomitantly, potassium tends to accumulate in the tissues, and sodium may be lost from the cells. Detailed analyses of muscle are given by Harrison and Darrow (1938), Darrow *et al.* (1939), Muntwyler *et al.* (1940), Conway and Hingerty (1946), and Cole (1950a).

If sufficient (but not excessive) amounts of salt and water are given to the deficient subject, and the dietary load of potassium is kept low, these changes can be largely reversed. The sodium otherwise lost is replaced, and with the large urine volume, potassium is carried out of the body in sufficient amounts to maintain normal ionic composition of both plasma and tissues (Harrison and Darrow, 1938).

Alternatively, administration of cortical steroids will induce retention of sodium and excretion of potassium and also restore normal tissue composition (Harrison and Darrow, 1938; Cole, 1950b; Davis *et al.*,

1951; Overman *et al.*, 1951). In this connection, it should be noted that the several naturally occurring steroids, as well as some of the synthetic products, differ considerably in their potencies in these respects. Deoxycorticosterone (a steroid which is secreted by the adrenal only in insignificant amounts) is very much more active in respect to retention of sodium than are the 11-oxycorticosteroids which constitute the bulk of the normal secretion. Aldosterone, although secreted in very small amounts, is so enormously active in sodium retention that it accounts for most of the sodium-retaining activity of the normal adrenal. In respect to potassium excretion, on the other hand, these steroids appear to differ much less widely in potency. Accordingly, the pattern of electrolyte effects seen in the plasma and in the tissues is likely to vary with the steroid administered and also with the dietary loads of potassium and sodium. A considerable confusion in the literature as to the relative effects of the several steroids on the composition of muscle may well have its origin in failure to consider all of these variables.

From the fact that salt therapy alone can be quite effective in restoring normal ionic composition to the muscle in adrenalectomized animals, it appears that the major factor affecting the tissue is the composition of the extracellular fluid, rather than direct effects of the adrenal hormones on the muscle cells. Cole (1950a, b), from detailed analyses of tissue and plasma, concluded that this was the principal mechanism in the action of deoxycorticosterone also. That cortical hormones do have extrarenal effects is uncontested, but these either are not demonstrably related to the movement of ions, or their importance in muscle is doubtful. Several groups of investigators (Gaudino and Levitt, 1949; Flanagan *et al.*, 1950; Hills *et al.*, 1953) have reported that the changes in the volume of intracellular and extracellular fluids occurring during the development of adrenal deficiency, or particularly after steroid therapy, are too large to be accounted for by changes in renal excretion of salts. If true, this could imply "sequestration" of salt in some noncirculating form in the body during deficiency, and withdrawal of the salt from this depot during treatment. This view has been contested by White (Netravisesh and White, 1953; White and Rolf, 1955), who could find no discrepancy between loss of salts during deficiency and retention during therapy in the dog, and no abnormal storage of salts in any of the tissues of adrenalectomized rats. In any case, however, such a "sequestration" does not appear to occur in muscle, since the sodium content of muscle water is low in adrenal

deficiency. Davis *et al.* (1951) and Overman *et al.* (1951) have reported that heavy dosage with cortisone induced some increase in muscle sodium without concomitant changes in serum sodium or sodium balance, suggesting a direct effect of the steroid on the muscle. On the other hand, Woodbury (1953) reported that although deoxycorticosterone or adrenocortical extract increased serum sodium in the absence of the kidneys, the sodium content of muscle either fell or did not change.

b. Potassium. With respect to potassium and water, extrarenal effects of the hormones are more evident than with respect to sodium. Cortical steroids have been reported to reduce the serum potassium without at the same time increasing its excretion, or even to do so in the nephrectomized animal. The fate of the potassium thus "retained" is not known. On the other hand, cortical hormones in excess, particularly the 11-oxycorticosteroids, have frequently been seen to induce losses of potassium from the body much too large to be due solely to the fall in extracellular potassium. The potassium content of the carcass (mostly muscle) of rats has been reported to be reduced when cortisone was given in quantity with or without extra salt (Knowlton and Loeb, 1947). The loss of potassium from the body has been correlated with changes in nitrogen balance, so that it is probably related to the loss of protein from the tissues which is seen regularly in these circumstances. As noted below, muscle is one of the major sites of the "catabolic" action of cortical hormones. Hence, the action of excess hormone on potassium content of muscle seems likely to be in large part the result of other metabolic events, rather than solely a direct effect on the movement of potassium ions from the tissue.

c. Water. The movement of water into the intracellular, or interstitial, compartment in adrenal deficiency and its withdrawal into the circulation on treatment with cortical hormones does not seem to be entirely explicable on the basis of changes in the ionic composition of the plasma. Gaunt *et al.* (1949) have reviewed the rôle of cortical hormones in water metabolism. Overhydration of the tissues of the carcass (muscle) of adrenalectomized rats was not corrected either by salt therapy adequate for survival and growth or by treatment with deoxycorticosterone (Knowlton and Loeb, 1947); nor is the failure of adrenalectomized subjects to excrete administered water improved much by either treatment (Gaunt *et al.*, 1949). As noted above, shifts

in the extracellular and intracellular water volumes have often appeared not to be explicable by alterations in salt balance; and large changes in extracellular fluid volume have been seen after the acute administration of cortical extracts to intact subjects, before significant renal retention of salts and water could have occurred (Bloodworth, 1952). Swingle *et al.* (1936, 1957), in observations of adrenal deficient dogs given cortical steroids but not salt or water, have shown convincingly that hemoconcentration can be abolished without alteration in the concentrations of electrolytes in plasma and even in the face of sodium diuresis. In all of these respects, the 11-oxycorticosteroids are much more active than is deoxycorticosterone. These observations indicate that the oxycorticoids, in addition to whatever effects they may have on plasma constituents, also may play an important part in controlling the distribution of water between intracellular and extracellular compartments, or between subdivisions of the extracellular fluid, independently of the ionic balances. The mechanisms concerned here are unknown, but Swingle suggests that they may be related to hemodynamic responses of the capillary bed.

2. *Carbohydrate Metabolism*

The cortical steroids do not seem to have any direct effects upon the metabolism of carbohydrate in muscle, although there are numerous indications of indirect or permissive actions of the hormones. When adrenalectomized animals are maintained in good condition by salt therapy or with deoxycorticosterone, the glycogen content of skeletal muscle is normal in the fed state, only slightly reduced if at all in fasting, and increases in approximately normal fashion after the feeding of carbohydrate (Anderson and Herring, 1940; Long *et al.*, 1940; Russell, 1940). The fasting respiratory quotient (R.Q.) is normal; and peripheral utilization of carbohydrate, as measured in the eviscerated animal, may be normal if the hemodynamic state is well maintained (Russell, 1943; Cohn *et al.*, 1952). When cortical steroids (11-oxy) are given to the fasting animal, the liver glycogen increases and blood sugar rises within a few hours, as is well known, but the muscle glycogen is increased only later if at all; so this is probably secondary to the hyperglycemia. Although large amounts of cortical steroids in fed animals may depress the R.Q. and induce a glycosuria which appears to be incommensurate with the increase in nitrogen loss, it has been difficult to obtain any more direct evidence of reduction in the oxidation or utilization of carbohydrate (Altszuler *et al.*, 1958, for review).

a. Relation to Other Hormones: Growth Hormone. On the other hand, cortical hormones seem to be able to affect the responses of muscle to some other hormones, particularly growth hormone but possibly also insulin and epinephrine. The first indication of this relationship was the observation that pituitary extract did not increase deposition of muscle glycogen or depress the R.Q. in adrenalectomized animals unless a small amount of cortical extract also was given (Russell, 1940). This effect has since been obtained with purified growth hormone (Illingworth and Russell, 1951). A similar relationship of cortical factors to the action of growth hormone has been indicated by many observations in the isolated diaphragm. Adrenalectomy, hypophysectomy, or both, tend to increase the response to insulin of the diaphragm of normal or diabetic rats (Krahl and Cori, 1947; Stadie *et al.*, 1949; Villee and Hastings, 1949; Bornstein and Trewhalla, 1950), and growth hormone, given *in vivo*, has been reported to reduce the glucose uptake, or the insulin effect on uptake, but only in the presence of some adrenocortical hormone either given or secreted *in vivo* (Park and Krahl, 1949; Park *et al.*, 1952; Stadie *et al.*, 1952). Cortical hormones alone have not generally affected diaphragm metabolism. Further, Bornstein and Park (1953) and Vallence-Owen and Lukens (1957) have reported on the presence in the serum of diabetic animals of a factor or factors which inhibited the glucose uptake or the insulin effect in normal rat diaphragm. The formation of these inhibitory substances appeared to require the secretion or administration both of adrenocortical hormone and of growth hormone. The mechanism of the permissive rôle of cortical hormone in respect to growth hormone effects on carbohydrate metabolism is quite unknown.

b. Insulin. Pretreatment of fasting animals with cortical hormone diminishes the hypoglycemic response to insulin, but this is largely the result of their glyconeogenetic action in the liver. Cortical extracts added to diaphragm *in vitro* have also been reported to inhibit the insulin effect (e.g. Clark, 1955); but since many steroids depress the metabolism of tissues *in vitro*, apparently in a nonspecific manner, these observations are not necessarily trustworthy. Levine *et al.* (1949) did not find any change in the sensitivity of the peripheral tissues of the adrenalectomized animal to insulin, and Persky *et al.* (1955) did not observe any reduction in insulin sensitivity on the acute administration of hydrocortisone. It is doubtful, therefore, if cortical hormones antagonize the action of insulin in muscle directly.

c. Adrenaline. In adrenalectomized animals, the hyperglycemic response to adrenaline has been reported frequently to be somewhat below normal, and the liver glycogen does not rise in the usual way. On the other hand, the increase in blood lactate after adrenaline is normal or somewhat elevated, and the fall in muscle glycogen has been found recently to be considerably greater in adrenalectomized rats than in normal animals (Collip *et al.*, 1936; Bloom and Russell, 1955; Winternitz *et al.*, 1955, 1957). The data of Winternitz and co-workers indicates that although the increase in liver glycogen in response to adrenaline could be restored readily with small amounts of cortical hormone, the abnormal depletion of muscle glycogen was affected only by intensive prior treatment. The response of the muscle glycogen to adrenaline in eviscerated adrenalectomized rats was not different from that of eviscerated adrenodemedullated controls. Winternitz considered that the reason for the abnormal depletion of muscle glycogen was the failure of the liver to form carbohydrate from the lactate released and so to return glucose to the muscles. However, before this may be taken as the sole explanation, one would wish to see similar observations made on intact adrenodemedullated animals; for it would not be unexpected to find the muscles of these subjects also hypersensitive to the glycogenolytic effect of adrenaline.

Cortisone given for several days was said to reduce the glycogenolytic action of adrenaline in the muscles of normal rats but to increase it in hypophysectomized animals (Wortman and Leonard, 1953; Leonard and Ringler, 1955); but the differences were of doubtful significance in either case.

Chronic treatment of rabbits with rather large amounts of cortisone has been reported to diminish the proportion of active phosphorylase in both muscle and liver (Kerpolla, 1952). On the other hand, Leonard (1957) did not see any effect of cortisone on the phosphorylase of rat muscle except that it appeared to reduce the extent of inactivation of the enzyme after stimulation of the muscle. The usual effects of adrenaline on the phosphorylase were not altered in either case.

3. *Protein Metabolism*

That the active 11-oxycorticosteroids tend to promote the catabolism of body protein is now well established from the work of many investigators. Adrenalectomized animals usually are unable to draw upon body protein to the extent seen in normal subjects under comparable

circumstances (e.g. in fasting, phlorizin diabetes, or trauma); and negative nitrogen balances are easily demonstrated with moderate to large doses of cortical hormone alone, or smaller amounts act in a permissive fashion for catabolic responses to other types of stimuli (Engel, 1951, 1952; Ingle, 1950, 1954).

From analyses of the tissues of animals given large amounts of cortical steroids, it is evident that the soluble carcass proteins contribute most of the nitrogen lost under the influence of the hormone (White and Dougherty, 1949; Kochakian and Robertson, 1951; Silber and Porter, 1953). The liver, on the other hand, often exhibits some degree of protein anabolism during the action of cortical hormone, presumably utilizing some of the amino acids from the periphery which are funneled through the liver as they are catabolized [references cited just above; also Roberts (1953) and Clark (1953)]. Although all or many other organs in the periphery may participate in the catabolic response, the skeletal muscles, because of their mass, must then constitute the major site of this effect.

A few more detailed studies have confirmed the action of adrenal hormones on muscle. The release of amino acids from the peripheral tissues in the eviscerated rat is slow in the absence of the adrenals and enhanced after administration of cortical hormones (Bondy, 1949; Ingle *et al.*, 1950b; Bondy *et al.*, 1954); and similar effects on the release of amino nitrogen have been seen in the diaphragm (but not the liver) incubated *in vitro* (Kline, 1949). The content of free amino acids in muscle *in vivo* has been reported to be increased in rabbits given cortisone or rats given corticotrophin (Dubreuil and Timiris, 1953; Sheffner and Bergeim, 1954). Also, the incorporation of isotopic glycine into muscle proteins of rats has been reduced, or its disappearance hastened, when the animals were given rather large amounts of cortisone or corticotrophin (Marshall and Friedberg, 1951; Clark, 1953; Fritz, 1956). The mechanism of this effect is not understood.

4. *Muscle Function*

From the relatively few detailed observations which have been made on muscle function in adrenalectomized animals, it appears that the defect responsible for the "myasthenia" is not in the initial contractile response of the muscle, but rather in the inability of the muscle to continue responding to repeated stimulation (Walker, 1947). In acute tetanic stimulation, the maximal tension is normal, but it may tend to

decline more rapidly (Winter and Knowlton, 1910) and fails altogether in later stages (Nicholson *et al.*, 1942). The phenomenon is best seen during and after brief periods of repetitive nontetanic stimulation (Everse and de Fremery, 1932; Walker, 1947) or in continued stimulation at slow rates which do not induce fatigue in the normal subject (Ingle, 1936, 1944; Goldstein *et al.*, 1950). In these conditions, the muscle of the adrenalectomized animal begins to fail quickly. The electrical properties of unfatigued muscle and nerve are normal, and the fatigue occurs just as readily with direct as with indirect stimulation (Walker, 1947), so that the defect does not appear to lie in conduction or transmission of the impulse.

The response of the cardiovascular system to pressor agents (sciatic stimulation, adrenaline and noradrenaline, or other pressor drugs) in adrenalectomized animals is much less than in the normal (Secker, 1949; Cleghorn *et al.*, 1950; Ramey *et al.*, 1951). From the conditions in which these observations were made, this failure does not appear to be due to chronic hypotension or other obvious signs of adrenal deficiency.

The nature of the therapy required to restore continued muscle function in adrenalectomized animals, and hence presumably also the nature of the critical defect, has varied with the experimental conditions. In all of the work on muscle mentioned above except that of Ingle and of Goldstein *et al.*, the animals were untreated, usually weak and listless, and may have been approaching adrenal crisis. In these conditions, pretreatment of the animals with deoxycorticosterone restored the response of the muscle (Everse and de Fremery, 1932; Nicholson *et al.*, 1942), whereas 11-oxycorticosteroids have been reported to be relatively ineffective (Waterman *et al.*, 1939). It may be supposed, therefore, that the principal controlling factor in these circumstances was the ionic composition of the plasma or of the muscle. This view is supported by the observations of Voegtli (1950) and of Ramey *et al.* (1950) that diaphragm or abdominal muscle from adrenalectomized rats responded normally to stimulation when suspended in normal Ringer solution. This was true of tissues taken from untreated "adynamic" rats, from those maintained with salt, or from animals exercised to exhaustion. On the other hand, normal diaphragms were quite hypodynamic when placed in a medium with sodium and potassium concentrations similar to those found in the plasma of animals in acute adrenal insufficiency (Voegtli). This behavior is not unexpected in view of the importance of these ions in muscle contraction.

That the ionic composition of the medium or tissue is not the only factor in the fatigue of adrenal deficiency is shown by the observations of Ingle and his co-workers and of Goldstein, Ramey, and Levine. In the extensive studies of Ingle (1944; Ingle *et al.*, 1945a, b; Ingle and Nezamis, 1948b), it was found that neither sodium salts in quantity nor deoxycorticosterone, given ahead of time or concurrently, were at all effective in prolonging the ability to perform continued work. On the other hand, the 11-oxycorticosteroids were quite active in this respect even when given acutely. The potencies of the latter steroids were closely proportional to their glyconeogenetic activities, but although their ability to maintain the blood glucose may have had some contributing effect, the provision of glucose alone was not helpful. Ingle has considered that the conditions of these experiments constitute a rather severe generalized body stress, and that the actions of the hormones here are probably systemic, rather than particularly upon muscle function.

In the work of Goldstein *et al.*, 1950, which was carried out on adrenalectomized dogs maintained with deoxycorticosterone, it was found that muscular responsiveness was closely related to the blood pressure, and that this could be restored by any means which also maintained the hemodynamic state. This relationship between circulation and the response of the muscles had also been demonstrated previously in acutely adrenalectomized and in intact cats by Schweizer (1947). From the work of Goldstein *et al.*, however, the critical defect in the adrenalectomized animal appeared to be its inability, as compared with the normal animal, to make the circulatory adjustments required to maintain its blood pressure during continued muscular activity. It was further shown by Ramey *et al.* (1951) that although the work performance of the adrenalectomized animal could be restored when the blood pressure was raised by noradrenaline, the pressor response to the latter hormone was less than in the normal animals, and that even this response could not be maintained for long. The response to noradrenaline could, however, be restored by the acute administration of adrenocortical hormones.

The same groups of investigators (Fritz and Levine, 1951) then studied the responses of the capillary bed of the rat mesoappendix to pressor agents. In the adrenalectomized animals maintained with salt, the capillaries were atonic, and although the initial constrictor response to noradrenaline applied locally was normal, repeated appli-

cations were progressively less effective and eventually caused irreversible atonia and stasis. The administration of oxycorticoids either systemically or locally restored the normal response of the capillary bed to the norepinephrine.

These observations suggest that the active oxycorticoids assist in the maintenance of muscle activity by allowing normal responsiveness of the circulatory system in conditions in which the blood flow must be shifted from one part of the body to another. They are consistent with the observations of Swingle *et al.*, mentioned earlier, in which the oxycorticoids brought about hemodilution in the adrenal deficient animal and restored normal vigor and activity without change in the ionic composition of the plasma.

Evidence for any more direct effect of cortical steroids on muscle function, in addition to those just described, is limited. There have been no convincing reports of changes in intracellular constituents of muscle which could account for the fatigue of adrenal deficiency. The acute intramuscular administration of large amounts of cortisone hemisuccinate to intact cats has been reported to increase the amplitude and maintenance of muscular responses to brief indirect stimulation (Del Pozo *et al.*, 1952). This is similar to the effect of adrenaline; it may have been due to reflex sympathetic stimulation, or it could conceivably be related to the reported effects of cortisone given chronically on the maintenance of active phosphorylase concentrations in stimulated muscle (Leonard, 1957).

E. Growth Hormone

The metabolic effects of growth hormone in muscle have been studied in normal and hypophysectomized rats, and, to a large extent in recent years, in the isolated diaphragm muscle. The effects observed have mainly to do with the carbohydrate metabolism of muscle, and these are not as yet clearly or immediately related to the growth-promoting actions of the hormone. The current picture of the effects of growth hormone on muscle is confused by the fact that, depending on the conditions of the experiments, the hormone sometimes seems to promote and sometimes to inhibit, the utilization of glucose by muscle. The inhibitory effects on occasion seem to be directly mediated by the hormone, but in certain other experiments the hormone appears to give rise to inhibitory substances which circulate in the plasma, so that the effect is indirect and takes time to develop. The details of much of

this work have been well reviewed by several workers (Krahl, 1951; 1955; Russell, 1953a; Ketterer *et al.*, 1957; de Bodo and Altszuler, 1957, 1958).

1. *Carbohydrate Metabolism in vivo*

One of the striking effects of hypophysectomy in the rat is that the glycogen of voluntary muscle disappears much more rapidly than normal during a fast, falling in 16 to 24 hours to about half the level of the fed state, which is usually not very different from that seen in normal fed rats. The cardiac glycogen, which rises during a fast in the normal rat, also falls to low levels in the fasting hypophysectomized rat. Since there is rapid depletion of the liver glycogen in the fasting hypophysectomized rat, it might be thought that the failure to maintain the muscle glycogen could be attributed to the early exhaustion of the hepatic sugar store and to the diminution of gluconeogenesis in consequence of the adrenal atrophy after hypophysectomy. In fasting adrenalectomized rats, however, the muscle glycogen stores are well maintained and the cardiac glycogen increases normally, despite the rapid loss of liver glycogen and the diminution of gluconeogenesis characteristic of these animals. The respiratory quotient of the hypophysectomized rat remains elevated during a fast. If the disposition of a carbohydrate meal is studied in such animals, it is found 4 hours after the meal, that there is less liver and muscle glycogen and a lower blood sugar, and that a much greater proportion of the glucose absorbed appears to be oxidized. Hypophysectomized rats with the basal metabolic rate restored to normal levels with thyroxine exhibit the same abnormalities, but the absolute rate of utilization of carbohydrate is still greater (Russell and Bennett, 1937; Russell, 1938a). In the eviscerated, functionally hepatectomized, hypophysectomized rat, the blood glucose and muscle glycogen fall more rapidly than normal. The rate at which glucose must be infused to maintain the blood sugar constant is about 25–27 mg. per 100 g. per hour as compared to 11–13 mg. per 100 g. per hour in the eviscerated normal rat (Russell, 1942). Similar rapid rates of glucose utilization were observed in hypophysectomized eviscerated rabbits by Greeley (1940). When it is recalled that the hypophysectomized animal, with its low level of thyroid activity, has a much lower metabolic rate than the normal animal, these differences are all the more remarkable.

If fasting hypophysectomized rats are treated with alkaline extracts

of anterior pituitary tissue (APE) or with purified growth hormone during the period of fasting, the fall in muscle glycogen is prevented and the cardiac glycogen rises rather than falls (Russell, 1938c; Russell and Wilhelmi, 1950; Illingworth and Russell, 1951; Adrouny and Russell, 1956). In fasting normal rats, the injection of growth hormone during the last 6 hours of a 24-hour fast also leads to a greater accumulation of glycogen in the gastrocnemius muscle, the diaphragm, and the heart (Illingworth and Russell, 1951). In these experiments, there were no significant changes in liver glycogen, so that the effects of growth hormone appear to be exerted chiefly in conserving muscle glycogen. Of the different muscles observed, the heart muscle appeared to be most sensitive to growth hormone, the diaphragm least sensitive.

The effect of growth hormone in conserving muscle glycogen in the fasting animal may be an example of a general tendency of the hormone to diminish carbohydrate utilization. Another aspect of this tendency may be seen from its effects on the disposition of fed carbohydrate in the intact rat. Fasted rats given a carbohydrate meal respond, during the 4 hours after feeding, with a rise in R.Q., and an increase in liver and muscle glycogen and blood sugar. If insulin is given with the meal, the rise in R.Q. is greater, there is a greater increase in muscle glycogen, and the liver glycogen and blood sugar are diminished. If APE or purified growth hormone is given prior to the meal, there is a still greater increase in muscle glycogen, the blood sugar and liver glycogen are about the same as in the normal rat, but the R.Q. does not rise nearly so high as after carbohydrate alone. Finally, if both insulin and growth hormone are given with the meal, there is a spectacular accumulation of muscle glycogen, the liver glycogen and blood sugar are intermediate between those seen in the untreated and the insulin-treated rats, and the R.Q. is still depressed as after APE alone (Russell, 1938b, d) Milman and Russell, 1950; Ulvedahl and Russell, unpublished). In experiments in another design, Illingworth and Russell (1951) also showed that insulin and growth hormone together had greater effects than either hormone alone on the glycogen of gastrocnemius, heart, and diaphragm of fasted rats given a glucose meal. All of these observations indicate that growth hormone affects the disposal of available sugar in favor of storage in the muscle rather than oxidation or conversion. It is interesting to note that, although the two hormones have opposing effects on the blood glucose, insulin and growth hormone work together in promoting the storage of glycogen in muscle.

Other aspects of the effect of growth hormone on carbohydrate metabolism are presented by the work of de Bodo and his colleagus on the hypophysectomized dog (reviewed in detail by de Bodo and Altszuler, 1957, 1958). The hypophysectomized dog in general behaves in a way quite similar to the hypophysectomized rat; the animals are highly sensitive to insulin, and, in the postabsorptive state, have much lower blood sugars than normal dogs. In experiments using C^{14}-glucose as a tracer, the interesting observation is made that in hypophysectomized dogs 16–18 hours after the last meal, the rate of uptake of glucose by the peripheral tissues and the rate of hepatic output of sugar are lower than normal. The rate of the CO_2 production in these animals is also much less than normal, as one might expect in hypophysectomized animals with much diminished thyroid function; one wonders, therefore, what portion of the total metabolism is contributed by the blood glucose, and what might be seen if these observations were made with hypophysectomized dogs brought to a normal metabolic rate with thyroxine. As they stand, however, these experiments give no evidence of an excessive continuing utilization of plasma glucose in the postabsorptive period. The injection of a small dose of insulin is followed by a greater than normal increase in the rate of uptake of plasma glucose by the peripheral tissues. The ensuing hypoglycemia is relatively even more severe, however, because the liver (which still contains adequate glycogen) fails to respond adequately to the falling blood sugar level. The exaggerated insulin sensitivity of these animals therefore has both a peripheral and a hepatic component. If hypophysectomized dogs are treated with growth hormone for 4–5 days, the level of the postabsorptive blood sugar rises, the rates of glucose uptake and inflow are restored to normal, the increase in glucose uptake induced by the same small dose of insulin used before treatment is reduced to normal limits, and this is now smoothly compensated by an adequate increase in hepatic output of glucose, an effect evidently brought about by growth hormone but as yet without explanation. In normal and adrenalectomized dogs also, treatment with growth hormone increases the level of the postabsorptive blood sugar and the rates of inflow and outflow of plasma glucose. In the conditions of these experiments, therefore, although growth hormone reduces the sensitivity to insulin, there is nevertheless an increased rate of flow of glucose from the liver to the peripheral tissues. An antagonism of growth hormone to insulin in the sense of a marked depression of glucose uptake by the peripheral

tissues is not seen. One does not of course know what is happening to the glucose after it leaves the plasma. Data on the specific activity of the expired CO_2 would be most interesting and enlightening in this respect. The picture of these events in the growth-hormone-treated dog is not inconsistent with the observations cited above on the glucose-fed rat treated with growth hormone or insulin and growth hormone. The novel feature of these observations is the effect of growth hormone on the hepatic output of glucose, but speculation on this point is outside the range of a chapter on muscle.

2. *Effects on Diaphragm and Heart in vitro*

In recent years, a great many observations have been made on the effect of growth hormone on carbohydrate metabolism in the isolated rat diaphragm (for reviews see Russell, 1953a; Krahl, 1955; Randle and Young, 1957; Ketterer *et al.*, 1957; de Bodo and Altszuler, 1957, 1958). The results of different investigators have varied a great deal in detail, partly no doubt because of differences in technique, sources of tissue, previous history and strain of animal, and differences in the nature of the hormone preparations used. It has generally been observed that diaphragm from hypophysectomized rats takes up glucose from the medium at a higher than normal rate, although this effect seems to be dependent in part on the glucose concentration in the medium; at the higher concentrations it is definite and reproducible. There is also general agreement, with one doubtful exception, that growth hormone added *in vitro* to diaphragm from normal or hypophysectomized animals does not depress glucose uptake or diminish the effect of added insulin in increasing glucose uptake or glycogen synthesis. Instead, many workers have noted that growth hormone added *in vitro* has itself an "insulin-like" action, increasing the rate of glucose uptake of diaphragm from hypophysectomized animals. An insulin-like action of growth hormone may also be observed in normal rat diaphragm, but it is dependent on the nature of the incubation medium (Park *et al.*, 1952; Ottaway, 1953; Bulbrook and Ottaway, 1954; Randle and Whitney, 1957). Russell (1953a) reported that growth hormone added *in vitro* to diaphragm from glucose-fed normal rats diminished the rate of fall of glycogen during incubation, but many subsequent attempts to repeat these experiments have so far failed (Russell, personal communication).

The injection of growth hormone into the animal prior to taking the

diaphragm is followed by an interesting series of events. If the tissue is taken within 3 hours, only an "insulin-like" effect on glucose uptake is seen, but thereafter, reaching a maximum in 18-24 hours, the glucose uptake and the response to insulin *in vitro* are much depressed (Park *et al.*, 1952). This delayed effect of injected growth hormone is enhanced by the injection of adrenal cortex extract. The absence of a direct effect of growth hormone *in vitro* and the development of a depression in glucose uptake in diaphragm removed from the animal some time after the injection of the hormone led to the suggestion that an altered form of the hormone, or some product released into the circulation after its injection, might be responsible for the altered behavior of the diaphragm toward glucose.

In pursuit of this suggestion, Bornstein and Park (1953) observed that the serum of alloxan diabetic rats contained a factor that inhibited the glucose uptake of normal diaphragm. The factor was absent from the serum of hypophysectomized alloxan diabetic rats, but it reappeared after the injection of growth hormone and adrenal cortex extract, but not if either hormone preparation was given singly. Their factor appeared to have the properties of a lipoprotein. Vallence-Owen and Lukens (1957) have made a similar set of observations on plasma from normal, depancreatized, hypophysectomized-depancreatized and adrenalectomized-depancreatized cats. The plasma insulin activity of all of the depancreatized animals, measured by the effect on glucose uptake of the rat diaphragm, fell to zero, and, in addition, there appeared in the plasma of the diabetic cats a factor which greatly inhibited the effect of added insulin (1000 microunits). The factor disappeared after hypophysectomy or adrenalectomy, and it was not restored by growth hormone alone or by adrenal cortex hormone alone. Since the inhibitory effect of the plasma from diabetic cats was not destroyed by freezing and thawing, it appeared doubtful that it could be a lipoprotein. The relation of this factor to that of Bornstein and Park remains to be determined.

Another interesting system in which an *in vitro* effect of growth hormone has been observed is the isolated perfused rat heart (Bronk and Fisher, 1957). This preparation has the advantages that it has a proper circulation to the cells, an active metabolism, and good sensitivity to both insulin and growth hormone. The rate of glucose uptake by the isolated heart, about 15 mg. per gram dry weight of heart per hour, is depressed about 50% by the addition of 0.1 μgm. per milliliter of growth

hormone to the perfusion medium. In the presence of 2 milliunits per milliliter of insulin, the rate of glucose uptake is 40 mg. per gram dry weight per hour; 0.1 μgm. of growth hormone per milliliter reduces this to 28–30 mg. per gram dry weight per hour. Studies with galactose indicated that growth hormone had the effect of diminishing the galactose space, whereas insulin has the effect of increasing it. An analysis of the time course of galactose penetration showed that growth hormone, in the presence or absence of insulin, approximately halves the numerical value of a derived coefficient which is taken as a measure of the activity of the carrier system responsible for the entry of glucose or galactose into the heart muscle cells. Insulin, on the other hand, in the presence or absence of growth hormone, increases the activity of the carrier system about fourfold. Since the actions of the two hormones are independent in the sense that each exerts its proportionate effect on the carrier system in the presence or absence of the other, growth hormone is not regarded as an "anti-insulin" but as a factor diminishing the scale of activity of the carrier system on which insulin acts. The authors point out that the observed effects of growth hormone and insulin on galactose penetration into the heart are entirely consistent with the observed relationship between the adenohypophysis and the pancreas: "First, the depressing effect of factor (1) (growth hormone?) does not involve insulin; it occurs in its absence. Secondly, the effect of insulin is not dependent on antagonizing factor (1): insulin is more effective in its absence. There is, in fact, an *in vitro* Houssay phenomenon in our system."

Bronk and Fisher use the term "factor (1)" because the growth hormone preparations used had more than one effect. The less pure preparation used had a complex, biphasic dose-response curve, in contrast to the more highly purified preparation derived from it. Both preparations became more effective on storage in dilute solution at —20°C. for several days, so that some labile factor acting in the same direction as insulin may also have been present. "Factor (1)" *may* be growth hormone, but a decision on this point is quite properly reserved. This interesting work on an active, intact muscle preparation needs confirmation and extension, but it shows great promise of providing a simplified system in which the analysis of hormonal actions may be carried out under more favorable, more nearly physiological conditions than *in vitro* experiments with thin muscle strips can permit.

3. *Summary*

The picture of the action of growth hormone on carbohydrate metabolism in muscle is as yet by no means clear. There is evidence that it acts to conserve the carbohydrate stores and that it influences the disposition of carbohydrate within the muscle. There are indications that, in the presence of insulin, the entry of sugar into the muscle may be facilitated (the "insulin-like" effect in the diaphragm), or that (as in the heart) it may act directly to limit the activity of the carrier system that is facilitated by insulin. The hormone also may give rise to substances (lipoprotein?) which can depress the uptake of glucose by the muscle. In the intact glucose-fed rat, the hormone depresses the R.Q. and spares the oxidation or conversion of glucose in the tissues. Whether this is a direct effect, or a consequence of the mobilization of an alternative substrate, fat, cannot yet be decided. There are many points of conflict apparent in the present observations, and it is not now possible to resolve them in terms of a single principle. It is evident that a great deal more work needs to be done, in a wider variety of conditions, and certainly on a wider variety of muscles, before a coherent picture of the action of growth hormone on muscle can be drawn.

F. Thyroid Hormone

That the muscles share in the general effects of thyroxine on the metabolic rate is well established. In the surviving tissues of hypo- or hyperthyroid animals, the muscles and heart have exhibited alterations in oxygen uptake of the same order as those seen in the intact animal, the cardiac tissue showing rather greater changes, skeletal and smooth muscle somewhat less (McEachern, 1935; Barker and Klitgaard, 1952). It is probable that the heart undergoes *in vivo* even greater alterations in metabolic rate than it shows *in vitro*; for in addition to the change in its own "basal" rate of heat production, the work required of the heart, in providing for the altered needs of the rest of the body, must be correspondingly affected. There has been no convincing demonstration that the thyroid hormone affects the metabolism or function of muscle in the mature animal in any other way than through its control of the metabolic rate and consequent effects. The mechanism of action of thyroid hormone on the metabolic rate has not been established (Barker, 1951).

1. *Metabolism of Foodstuffs*

The metabolism of carbohydrate and fat in muscle seems to undergo

no special alteration with deficiency or excess of thyroxine other than those expected from the relationship of the supplies of foodstuffs to the demands for energy. The rates of catabolism of administered carbohydrates and ketone bodies and of body fat are affected about in proportion to the metabolic rate, whether it is high or low, and the proportionate disposition of fed carbohydrate in muscle is normal in hypothyroidism. In hyperthyroidism, the glycogen stores of muscle may be depleted, especially in the heart; but the fall in the cardiac glycogen has appeared to be related quite closely to the work of the heart as indicated by the pulse rate, and similar effects on glycogen have been produced by other means of altering the heart rate (Moses, 1942). It is probable that similar relations of supply and demand obtain in skeletal muscle and liver as well.

2. *Protein Metabolism*

The relationship of the thyroid hormone to protein metabolism is biphasic. On the one hand, as mentioned earlier (p. 144), small amounts of thyroxine are necessary for normal growth and development, so that in this sense the hormone is anabolic. On the other hand, the "basal" rate of protein catabolism is correlated directly with the basal metabolic rate. This has been seen in the nitrogen excretion during fasting or in subjects on nitrogen-free diets (the "endogenous" nitrogen catabolism); in the rate of release of amino acids from peripheral tissues of eviscerated animals (Bondy, 1949); and in the release of protein and amino acids into the medium from isolated diaphragm (Kline, 1949). When fasting thyroidectomized rats were given N^{15}-glycine, a larger proportion of the isotope was excreted and this was diluted to a lesser extent by endogenous unlabeled nitrogen. Hence, both the anabolism and catabolism of body protein appeared to have been diminished (Hoberman, 1950). Similar observations in patients with myxedema before and after treatment with thyroid hormone also indicated that the rates of synthesis and breakdown of body protein were both affected in parallel (Crispell *et al.*, 1956). The rate of weight loss in denervated muscle was slower than normal in thyroidectomized animals, but occurred at the normal rate when the animals were given thyroxine in amounts which restored the normal metabolic rate (Hines and Knowlton, 1934). The effects of thyroid hormone on the net rate of protein catabolism evidently did not require innervation and were not dependent on muscle work.

In both clinical and experimental hyperthyroidism, a negative nitrogen balance is frequently seen; and if the condition is prolonged, a considerable degree of wasting of the muscles and other tissues can occur. Since the negative nitrogen balance can be overcome by the provision of sufficient food, it is probably mainly the result of imbalance between supplies of foodstuffs and the increased metabolic rate. One may suppose that if anabolism of protein is increased in this state, it is augmented to a lesser degree than is the rate of catabolism. An increase in the requirements for and secretion of adrenocortical hormones, which has been postulated to occur in hyperthyroidism, might be expected to enhance the tendency to lose nitrogen in this condition.

3. *Creatine Metabolism*

Because of the weakness of the muscles characteristic of hyperthyroidism, much attention has been devoted to creatine metabolism in relation to the thyroid hormone. The subject has been reviewed extensively by Wang (1939). Creatinuria, diminished creatine tolerance, and slightly lower excretion of creatinine are usual in both clinical and experimental hyperthyroidism. The total creatine content of the muscles, particularly of the heart, has been reduced in thyrotoxic animals and the phosphocreatine content also is said to be low (Wang, 1939; Shelley *et al.*, 1943). In skeletal muscle, the reduction in phosphocreatine was the same as that in total creatine in absolute terms, but larger proportionately; but the changes were rather small in any case (Wang, 1939). It may be remarked that in nearly all of the work of this type done in animals, the dosages of thyroid hormone used have been massive in the extreme—i.e., just sublethal or sometimes even fatal in amount. The changes so induced in creatine and phosphocreatine and other energy sources may be more expressive of nonspecific damage than they are of thyroid hormone activity on muscle metabolism per se. From this work, no special alterations in creatine metabolism or in the functional aspects of creatine in muscle seem to have been uncovered. Since creatinuria and loss of creatine from muscle are seen in a variety of conditions in which atrophy or wasting of the musculature is prominent, it is probable that in hyperthyroidism also these features are related mainly to the loss of substance from the muscles. The relative depletion of phosphocreatine and other energy sources might be expected under conditions of relative overwork, as in the thyrotoxic heart.

4. *Muscle Function*

Despite the fact that weakness of the voluntary musculature and other functional myopathies are common in hyperthyroidism, remarkably little work has been done on the fundamental nature of the functional effects of excess or deficiency of thyroid hormone in muscle. In a careful study of the Achilles tendon reflex in man, Lambert *et al.* (1951) confirmed the common clinical impression that the contraction is slow in most patients with hypothyroidism and tends to be accelerated in hyperthyroidism. There was no change in the interval between stimulation and the onset of contraction; but both contraction and relaxation were affected, the latter to a greater extent than the former. This suggests that the *alterations lie within the muscle, rather than in* conduction or transmission.

There have been many reports that the efficiency of muscle work is low in hyperthyroidism—that is, that the increase in oxygen uptake is large in relation to the amount of work done during exercise. Schwartz and Lein (1955) have studied the characteristics of muscle contraction simultaneously with oxygen consumption in hypothyroid or moderately hyperthyroid rats. When the rate of stimulation was relatively low (40 per second), the steady-state tension was low in hyperthyroid and high in hypothyroid animals, and the ratio of excess oxygen use to the tension was altered inversely. It was noted, however, that the fusion of responses at this frequency was quite incomplete in the hyperthyroid animals, complete in the hypothyroid, and intermediate in the controls. This is consistent with the apparent changes in contraction time, a muscle displaying rapid contraction and relaxation, as in hyperthyroidism, being expected to have a high fusion frequency. At higher rates of stimulation, in which fusion was complete in all animals, the steady state tension, although somewhat low in the hyperthyroid animals, was not regularly related to metabolic rate, and the ratio of oxygen use to tension did not differ significantly from normal in any group. From this work, it appeared that the thyroid hormone did not affect muscle economy directly, but that the apparent efficiency of the muscle could be altered by changes in contraction time and degree of fusion of responses to stimulation.

Additional factors which may affect muscle function in hyperthyroidism are, perhaps, the wasting of the tissues in the chronic state, and more important, the probable difficulty in assuring adequate oxygenation in the face of the very great demand which may occur in local

areas during exercise. The relative importance of all of these factors in the hyperthyroid state remains to be assessed.

In hypothyroidism, muscle function, although slow, does not seem to be much affected otherwise. The myotonia sometimes described could well be the result of slow relaxation and diminished fusion frequency.

G. Parathyroid Hormone

Although it is well established that the disturbances in excitability that are seen in states of hypo- or hyperparathyroidism are referable to the effects of an altered calcium ion concentration on the nervous system or the neuromuscular junction, there is also the possibility, less clearly recognized but supported by a few observations, that the parathyroid hormone may exert an effect on muscle itself. Brown and Imrie (1932), studying the fall in the excretion of urinary phosphate that follows the infusion of creatine into decerebrate cats, observed that the injection of parathyroid hormone, either 18 hours before or immediately before the infusion was started, brought about a greater and more prolonged fall in the excretion of urinary phosphate. The injection of the hormone alone was followed by an increase in phosphate excretion, as one would expect. There was also no effect of the hormone itself on the concentration of phosphocreatine or any of the other phosphate fractions of muscle, but when both creatine and parathyroid hormone were given, marked temporary increases in muscle phosphocreatine were observed.

These observations were carried further by Imrie and Jenkinson (1933), who observed that in thyroparathyroidectomized cats, decerebrated, or anesthetized with chloralose, the muscle phosphocreatine is lower than normal, falls to lower levels after stimulation (through the ends of the nerves, previously cut), and recovers only very slowly. Treatment with parathyroid hormone (10 units, twice daily for 2 to 4 days) brought about an increase in phosphocreatine and restored to normal the rate of recovery of the phosphocreatine after stimulation. The authors concluded that parathyroid hormone has an influence on the maintenance of phosphocreatine in the muscle, but they made no comment on the possible mechanism of this effect.

More recently, additional indirect evidence has been obtained which suggests that the parathyroid hormone may have an effect on movements of phosphorus in the body other than those which result from the

actions of the hormone on the kidney and the bones. Howard *et al.* (1953) observed that, if calcium salts are given intravenously to normal individuals (which it is thought would suppress the output of parathyroid hormone) there is a rise in serum P that is not accounted for by the retention of P by the kidney. The suggestion was that there had been a shift of P from the intracellular to the extracellular compartment. To this may be added the observations of Milne (1951), who found that in hypoparathyroid subjects, the extra P excreted by the kidney in response to the injection of parathyroid hormone did not account for the P which disappeared from the extracellular compartment. There may therefore have been a shift of P into the soft tissues in response to the hormone. Finally, in the course of attempts to develop an assay for parathyroid hormone in rats, Tepperman *et al.* (1947) observed that the direction of change in the serum inorganic P after injection of the hormone depended on the state of the animal and the route of the injection. In fasting rats, no change in serum P occurred after subcutaneous injection, but a prolonged rise in serum P was observed after intraperitoneal injection of the hormone. In the fed animal, however, intraperitoneal injection was without effect, but subcutaneous injection was followed by a fall in serum P, maximal in 2 hours, which was proportional to the dose of hormone injected.

All of these observations suggest that the parathyroid hormone may have effects on tissues other than bone and kidney, and that, since these effects seem to be concerned with the transfer of phosphate, the effects on muscle metabolism and function may be interesting and important. The probability that much better preparations of the parathyroid hormone may soon be available makes the renewed investigation of this aspect of the function more attractive. It would be interesting, for example, to learn whether the fall in serum inorganic P which may be induced by carbohydrate feeding is in any way influenced by parathyroidectomy or by the parathyroid hormone.

H. Ovarian Hormones

In addition to their effects on uterine growth and on the development of the endometrium, the ovarian hormones (estrogen and progesterone) influence the functional behavior of the uterine muscle. An excellent account of the actions of the ovarian hormones on uterine muscle is given by Csapo (1956). The following brief summary is taken largely from his paper, which may be consulted for details and for additional references to the literature.

During estrus, the spontaneous uterine contractions tend to be large and infrequent; in the luteal phase of the cycle, when progesterone dominates the uterus, contractions are very frequent but small. The estrogen-dominated uterus is sensitive to oxytocin, but the progesterone-dominated uterus is refractory to the posterior pituitary hormone. Progesterone therefore tends to block the contractile activity of the uterus, inducing a state of relative quietude which is evidently part of the general protective role of the corpus luteum hormone in the maintenance of pregnancy. The mechanism of this effect is not yet certainly established. It has been shown that the maximal contractile capacity of the myometrium is unaffected by progesterone, so that the blocking action is not exerted upon the contractile system itself, but is more likely associated with the processes of excitation and conduction. A study of the electrolytes of the uterine muscle reveals that the estrogen-dominated uterus has a high intracellular potassium concentration, and low intracellular sodium, the ratio K/Na being 5.3. The progesterone-dominated uterus, however, has a low potassium and a high sodium concentration, the ratio K/Na in the muscle being only 2.9. Since the extracellular potassium and sodium is unaltered in the two states, the effect of these changes is to alter the electrochemical gradients between the uterine muscle cells and their surroundings. The effect of progesterone is therefore to reduce the membrane potential on the one hand, facilitating local activity, but on the other hand to diminish the propagation of the contractions (an effect of the increase in cell sodium) so that massive full-scale contractions are interdicted. The restoration of the stable but fully responsive state characteristic of estrus can occur rapidly after the withdrawal of progesterone because of the ease and speed with which the intracellular ion concentrations may be adjusted. The mechanism by which progesterone brings about the changes in distribution of potassium and sodium is as yet unknown.

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CHAPTER V

The Neuromuscular Junction

Role of the Acetylcholine System[1]

DAVID NACHMANSOHN

I. INTRODUCTION

The last few decades have seen remarkable progress of our knowledge of nerve function. The use of intracellular microelectrodes for the study of electrical manifestations, the availability of radioactive material for measuring ion movements, the use of monocellular preparations, improved methods for recording heat production, to quote some of the most important inprovements, have yielded much new information about the physical events taking place during nerve activity. Developments of biochemical aspects were just as striking: the elucidation of metabolic pathways, the rapid progress of protein and enzyme chemis-

[1] Some of the material in this Chapter has been presented in: D. Nachmansohn, "Chemical and Molecular Basis of Nerve Activity," Academic Press, 1959.

try, the analysis of molecular forces acting in enzymes and their relation to function have greatly advanced our knowledge of the chemical basis underlying cellular function in general and propagation of nerve impulses in particular. Finally, histochemical studies and electron microscopy have provided pertinent new information of ultrastructure of cells and of cellular organization of chemical reactions in relation to function.

The transmission of nerve impulses across synaptic and neuromuscular junctions is only a special aspect of the primary function of nerves, which is the propagation of nerve impulses. Manifestations of activity at these foci differ in some respects from those of conduction along axons. *The aim of this article is to present our knowledge of the properties of the acetylcholine system and to discuss its rôle in the transmission of nerve impulses across the neuromuscular junction.* Before we can discuss intelligently and profitably the special aspects of transmission across synaptic and neuromuscular junctions, we must review some pertinent facts and concepts concerning the mechanism of conduction of nerve impulses in general. In the light of this information, differences, or similarities, of the properties and function of the acetylcholine system in the propagation across junctions will be evaluated.

II. EVENTS IN THE AXON DURING NERVE ACTIVITY RECORDED BY PHYSICAL METHODS

A. The Membrane Theory

It has been known for nearly a century that small electric currents propagate nerve impulses along axons. However, as was recognized by the physiologists of the last century, the electrical manifestations are only one part of the activity; they do not reveal the underlying mechanism. All modern concepts as to the mechanism of conduction are still based on the so-called membrane theory. When in the latter part of the last century it became known that semipermeable membranes may develop strong potential differences, physical chemists like Traube, Ostwald, Nernst, and others, developed notions and theories which form the basis of the membrane theory, best known through the formulation of Bernstein and Tschermak early in this century (Bernstein, 1902). According to this theory, the nerve fiber is surrounded by a semipermeable membrane which has a positive charge on the outside and a negative one on the inside. It is selectively permeable for K^+. When a stimulus reaches a membrane, the permeability at the active

site is greatly increased for all ions with a concomitant decrease in resistance. The active part becomes depolarized; thereby small electric currents are generated which stimulate the adjacent points and the same process takes place there. In this way, successive parts of the membrane are activated and the impulse is propagated along the axon.

It is a remarkable tribute to the ingenuity of the physical chemists of the last century that in spite of all great progress of methods and knowledge during this century, the membrane theory has remained the basis of our present concepts. Only one modification has become necessary. At the activated point there is not just a simple depolarization but a reversal of polarity; the inside becomes positive and the outside negative. This has been shown by Curtis and Cole (1942) and by Hodgkin and Huxley (1945) by insertion of electrodes into the interior of the axon (the giant axon of squid was used) and by direct measurements of the potential between the inside and the outside electrode. During the passage of the impulse the charge does not just disappear, as was assumed originally, but is reversed. By this overshoot the action potential becomes about twice as great as the resting potential.

An important advance was the experimental evidence by Cole and Curtis (1939) for a breakdown of resistance during activity. According to their measurements, which were carried out on the giant axon of squid, the resistance during activity drops from about 1000 to 40 ohms cm^2.

B. Ion Movements

In the interior of most cells, ions are present in concentrations quite different from those in the extracellular fluid. Na^+ on the outside is usually about 10 times as high as inside the cell, the reverse is true for K^+. Sometimes the differences are even higher. In a fluid system, ions must be the carrier of electric currents and it has always been assumed that ionic concentration gradients are the source for the EMF of the electric potentials. In other words, the conducting cell makes use of the uneven ion distribution for the special purpose of generating small electric currents. Overton (1902) recognized the importance of sodium for excitability. He suggested that conduction was associated with an exchange of extracellular Na^+ with intracellular K^+. The availability of radioactive ions after the second world war made it possible to measure quantitatively ion movements in rest and during activity. The

first measurements of this type were carried out in 1947 by Rothenberg in my laboratory. Using the giant axon of squid he found that both Na^+ and K^+ move across the membrane in rest; the equilibrium is dynamic (Rothenberg and Feld, 1948). During activity, there takes place a sudden influx of Na^+ and an equivalent amount of K^+ leaks to the outside. The amount of Na^+ per cm^2. per impulse was estimated to be 4 $\mu\mu$moles (Rothenberg, 1949, 1950). This figure was later confirmed by experiments of Hodgkin and Huxley and their associates in Cambridge. Keynes and Lewis (1951) demonstrated that an equivalent amount of K^+ leaks to the outside. The analysis of the ionic movements during activity has been extended by the Cambridge group with greatly refined methods based on principles worked out by Cole (1949, 1955) (Hodgkin, 1951, 1957; Huxley, 1954). Their investigations have shown that during activity there is at first a specific rapid transitory increase of permeability to Na^+. This change apparently permits Na^+ to enter during the rising phase of the action potential. The permeability for this ion species during the active phase was estimated to be about 500 times as high as in rest. The movement of Na^+ into the interior makes the inside positive. During the descending phase, K^+ moves to the inside. The current carried by Na^+ very rapidly reaches its peak and falls nearly just as fast; that carried by K^+ rises more slowly in an S-shaped curve and reaches a level maintained with little modification until the resting condition is restored.

The rôle of calcium during activity has also been investigated. The spontaneous activity of nerve fibers in Ca-poor Ringer solution has been known for a long time. When a squid fiber conducts impulses, a small quantity of calcium enters (Flückiger and Keynes, 1955; Hodgkin and Keynes, 1957); the quantity per cm^2. per impulse is only 0.006 $\mu\mu M$ or 1/700 of that of Na^+. Hodgkin (1957) considers as one possible explanation that the movements of calcium have something to do with the change of permeability; the local electric field inside the membrane may be altered by absorption of calcium ions to the membrane, without changing the overall potential difference between external and internal solutions.

C. Heat Production

The great number of messages which the nervous system continuously carries throughout the organism makes it nearly imperative to perform this function in an economic way, i.e. with a minimum expense of

energy. Nature has indeed developed a mechanism which requires a really amazingly small amount of energy for the primary event, as indicated by the small amounts of heat production. Their demonstration required the development of extraordinarily sensitive recording instruments. A. V. Hill and his associates were the first to be able to measure amounts of heat of such a small order of magnitude. The results have been well summarized and evaluated by Hill (1932a, b) and by Feng (1936). Only a few figures may be mentioned as an illustration. The greatest rate of heat production in frog sciatic nerve due to steady stimulation (with frequencies below 50 per second) is of the order of magnitude of 40×10^{-6} cal. per gram nerve per second. At low frequencies, the total yield per impulse is about one microcalorie per gram, at 19°C.

As in muscle, one may distinguish two phases: the initial heat, closely associated with activity, and the delayed or recovery heat. Whereas in muscle the two phases are of a similar order of magnitude, the initial heat produced during nerve activity is extremely small. In frog nerve at 20°C., the initial heat is about 7×10^{-8} cal. per gram per impulse, at 0°C. it is about 4 times as high (26×10^{-8} cal.). The surface per gram nerve in frog sciatic is estimated to be about 1600 cm^2. The initial heat per cm^2. at 20°C. would then be about 4×10^{-11} cal. per impulse. In *Maja* nerve, the initial heat per gram per impulse is 0.73×10^{-6} cal., at 16.5°C. Thus the initial heat in crab nerve at this temperature is about 3 times as great as that of frog nerve at 0°C. and at least 10 times as great as that at 20°C.

The initial heat appeared so small that the question has been raised whether a chemical reaction could be responsible for its origin. It was suggested that the exchange of Na^+ and K^+ between axoplasm and outside fluid may account for it (Hodgkin, 1951). In this view, the generation of bioelectric currents does not require a chemical reaction, but may be effected by a purely physical process.

Recently, significant new information about the initial heat has been obtained by A. V. Hill and his associates (Abbott *et al.*, 1958) with still more rapid recording equipment than was previously available. The initial heat production of a non-medullated nerve (*Maja*) has been separated into two successive phases. In the first phase, there is a positive heat production, averaging in a single impulse at 0°C. about 9×10^{-6} cal. per gram nerve; this phase is rapid and is probably associated with the active phase of the impulse. In the second phase, lasting for about 300 msec., heat is absorbed, averaging about 7×10^{-6} cal.

per gram nerve. With the previous methods, only the difference between the two processes, the "net heat," namely about 2×10^{-6} cal. per gram, was recorded.

Maja nerves contain fibers from 20 to 0.3 μ in diameter. About half the heat is probably derived from fibers less than 3 μ. The conduction velocities at 0°C. vary from 1.4 to 0.1 meters per second; impulses reach *the recording thermojunctions throughout at long intervals.* The observed heat production is the resultant of positive and negative components in different fibers and a substantial part of each is masked. The real positive and negative heats, therefore, are substantially greater than those observed. On the most likely estimate of velocity distribution, the authors arrived at values of 14×10^{-6} cal. and -12×10^{-6} cal. per gram per impulse, at 0°C.

For the evaluation of these figures, it is important to realize that the process of nerve impulse conduction is generally believed to be a surface phenomenon; the ions are assumed to move across a membrane of about 50 A. thickness. The observed permeability change must take place in this layer and so should chemical events, if associated with this change. The structural organization of chemical processes and their significance will be discussed later. The surface in 1 g. *Maja* nerve is estimated to be 10^4 cm^2. Referring the heat to 1 g. surface material instead of to 1 g. nerve, Hill and his associates arrive at the amazing amount of 2.8×10^{-3} and -2.4×10^{-3} cal. per impulse, which is about the same order of magnitude as the heat produced per gram in a muscle twitch.

What then is the origin of the heat production? In his lecture on "Chemical Wave Transmission in Nerve," A. V. Hill (1932b) considered the possibility of energy liberation in the active membrane in which large changes of permeability are produced and reversed during activity. In the recent paper, the authors write, "It is difficult indeed to imagine an excitable membrane going through a complete cycle involving a several hundredfold increase in permeability to Na^+ ions followed by a similar increase in permeability to K^+ ions, and yet behaving as a conservative system without change of energy. . . . It is hard to believe that so drastic a cycle of physicochemical change could occur in material like that of the excitable membrane without the intervention of work or chemical reaction."

Among some explanations discussed by Hill and his associates is the possibility that the positive heat is derived from the energy released

during the rising phase of the action potential, in the discharge of the condenser which exists all over the excitable membrane. The negative heat would then be due to the absorption of energy in recharging the condenser during the falling phase. This "condenser theory" encounters the difficulty that the time relations seem to be wrong, as pointed out by the authors.

The heat of ionic interchange can at present be derived only from very indirect types of information. The quantities involved are much too small for direct measurements in a calorimeter. The actual conditions under which the exchange in nerve takes place are quite different from those of the experimental measurements from which the values have been estimated and extrapolated to the physiological event. Emphasizing these uncertainties, the authors try to evaluate the various available previous data on ion flux and additional new data obtained on *Maja* nerve under conditions as similar as possible to those of their heat experiments. They arrive at a figure which amounts to about half of the initial positive heat.

A fact pertinent for overcoming this and other uncertainties in the interpretation of the origin of the heat is the absence of any marked effect of temperature on the net heat. The initial positive and negative heats cannot be evaluated at higher temperatures, since at present, instruments are not quick enough to separate them. The near independence of temperature favors the theory that the net heat is due to chemical reactions involved in the permeability cycle; for if the cycle is the same, though occurring quicker at a higher temperature, the reactions would probably be the same. For the condenser theory, the temperature effect has no bearing, since the net heat cannot be attributed to condenser discharge and recharge. For the theory attributing the net heat to the interchange of sodium ions and potassium ions, the small temperature effect is unfavorable, for the amount of interchange observed is much greater at a lower temperature. "For this reason," the authors write, "it seems very unlikely that the net heat could be derived from the ionic interchange alone."

D. Temperature Coefficient

For the question whether purely physical processes may be assumed to be responsible for the action potential, another type of measurement is pertinent, namely the temperature coefficient. There are relatively few data reported in the world literature about the Q_{10} of bioelectric

currents. Hodgkin and Katz (1949) did find high temperature coefficients, but this finding was not considered as relevant to the problem of the nature of the conducting process.

Recently, Schoffeniels (1958b) has evaluated the Q_{10} and the energy of activation of bioelectric potentials over a wide range of temperature, using the isolated single electroplax of the electric organ of *Electrophorus electricus*. He determined the duration of the action potential of the electroplax, of the postsynaptic potential, and of the latency period of synaptic transmission as a function of temperature. He found that the duration of all three phenomena decreases with rise of temperature, whereas the amplitude of the spike in the postsynaptic potential remains unchanged. Since during the action potential, there is a marked transitory change of permeability, the duration of the spike is a good measure of this change and pertinent for the question whether or not chemical reactions are required to this process. A straight line was obtained when the logarithm of the reciprocal of the half width of the spike was plotted against the reciprocal of the temperature according to the principle of Arrhenius. The Q_{10} of the action potential was found to be around 3.6 and the energy of activation to be 21,000 cal. per mole. The Q_{10} of the latency period and of the postsynaptic potential are close to 2.6 and the energy of activation around 16,000 cal. per mole.

E. Problems Resulting from the Measurements of Physical Events

The recordings of physical events have provided important information about electric potentials in rest and during activity, the change of resistance during the passage of the impulse, and the magnitude of energy transformations. The analysis of ionic movements established the sequence of changes of Na^+ and K^+ conductance. But physical recordings cannot explain mechanisms of living cells. More than one hundred years ago, Justus von Liebig wrote, in the introduction to his "Thierchemie," that no manifestation of life is conceivable without a chemical reaction and that the knowledge of molecular forces is a prerequisite for any understanding of cellular function. Similar views were repeatedly expressed by Hopkins, Meyerhof, and by other leading biologists of our time. It is inconceivable that electricity in a fluid system, i.e. in a living cell, can be generated without a chemical reaction. The view that chemical reactions must take place in the elemen-

tary process of nerve conduction has now found experimental support by the biophysical data on the initial heat production, temperature coefficients, and activation energies.

This bring us to the question, what is the mechanism underlying the generation of bioelectric currents? More precisely, what chemical reactions are responsible for the sudden transitory change in permeability to sodium and how do they control the ion movements during activity? What is the trigger by which the potential source of energy, i.e. the ionic concentration gradient, becomes suddenly effective? Do we have a clue to the molecular processes taking place in this event? A real understanding of nerve activity is only possible by integration of the physical events with the underlying chemical processes.

A clear distinction must be made between the events taking place during activity and those in recovery. The flux of Na^+ and K^+ during activity is in the direction of the concentration gradients. The suddenly increased rate of flow for the extremely short period of time (less than 1 msec., according to physical recordings) apparently requires very little energy for effecting the transient change of permeability. The problem is fundamentally different in respect to ion movements during recovery. The restoration of the original steady state requires extrusion of Na^+ and uptake of K^+ against the concentration gradient. The maximum rate at which the ions are extruded against the gradients is about 50 $\mu\mu M/cm.^2/sec.$, while during activity the movements may reach a rate of about 10,000 $\mu\mu M/cm.^2/sec.$ (Hodgkin and Keynes, 1955). The movements against the gradient require relatively large amounts of energy in excess of that necessary for maintaining the resting condition. Most of the extra heat produced is accordingly developed after the electrical changes and is presumably associated with chemical reactions required for the restoration of the initial electrolyte distribution. The energy for the ion transport must be provided by chemical energy probably derived from reactions common to most cells. There is no disagreement on this point. The sudden movements of Na^+ and K^+, on the other hand, carrying bioelectric currents, are a very specific phenomenon of conducting cells. Here one would suspect *a priori* a chemical system specific for conducting cells which effects the sudden permeability changes in the membrane or in the special molecular layer responsible for the permeability change. The problem of the properties of the membrane has been strongly emphasized by Kurt H. Meyer and T. Teorell. Meyer (1937) postulated on the basis of experiments with

monomolecular films that a chemical reaction in the active membrane must precede the change in ion permeability. Membranes are formed by chains of protein (or conjugated protein). Meyer assumed that a chemical reaction may result in a rearrangement of acidic and basic groups; positively charged NH^{+} groups would increase anion permeability, and COO^{-} groups would facilitate the movement of cations. The importance of the rôle of charges in the membrane has been stressed for a long time by Teorell. In recent publications (Teorell, 1951, 1953), he has extensively scrutinized the theoretical basis of the permeability problem.

The difficulty of identifying and analyzing the chemical reactions generating the electric currents is easily recognized if one considers the extremely small amounts of heat produced, an indication of the smallness of the chemical reactions. Moreover, as the impedance changes indicate, the processes take place within a fraction of a millisecond. Obstacles of a considerable magnitude were encountered even in the recording of the physical events with highly sensitive instruments. However, the spectacular progress of physics and chemistry has provided for the biologist extremely powerful tools for the analysis of cellular function. A familiar example of the great progress achieved by biologists in this century is the knowledge obtained about the elementary processes of muscular contraction. Hardly any other problem has attracted so many outstanding physiologists and biochemists during this century. The challenging problem in this case is the question, how a cell is able to use the energy of chemical reactions for performing mechanical work. The large amount of energy involved and the convenience of measuring the work performed obviously offered to the biologist a particularly favorable material for an attempt to explain cellular function in terms of physics and chemistry. A special rôle in this development must be attributed to Otto Meyerhof. The principles and notions which he emphasized and applied had a great influence on contemporary biochemistry and biology. They became an integral part of studies of cellular function in general and form the basis of the approach presented in the following to analyze the elementary processes of the propagation of nerve impulses. The challenging question in this case is, how chemical reactions generate bioelectric potentials. In spite of the fundamental differences between these two cellular functions, the experience gained in the efforts to explain transformations of chemical into mechanical energy, to correlate metabolism and

function in the one type of cell, has been frequently invaluable for the interpretation and evaluation of the information obtained in studies of the other type of cell.

III. HYPOTHESIS OF NEUROHUMORAL TRANSMISSION

At about the same period in which Du Bois Reymond's classical work laid the basis of electrophysiology, Claude Bernard, in a series of remarkable papers, published his observations on the effect of curare; this poison blocks the transmission of nerve impulses to the muscle although the conduction in nerve and muscle fibers remains unchanged. These observations marked the beginning of the notion that the junction between nerve and muscle has peculiar properties. This view was supported by a number of developments. Histological examinations showed a structural differentiation of the effector cell at the junction, the motor end-plate. The neuron was found to be a cellular unit and it was established that between nerve and muscle and between nerve terminal and nerve cell body, i.e. at the synapse, there is a close contact but no protoplasmic continuity. This notion obviously raised the problem of how nerve impulses were propagated across these junctions.

Physiological observations seemed also to indicate characteristic and special properties of these junctions. Some of the most important may be briefly mentioned: (1) Propagation of nerve impulses from nerve to muscle and across synapses is effected in one direction only. (2) Fatigue of a nerve muscle preparation often occurs at the junction when stimulation of nerve and muscle fiber does not yet indicate any alteration of its functional ability. (3) A synaptic delay is observed when impulses are propagated from one cell to the other. (4) Junctions are much more sensitive than fibers towards all kinds of physical and chemical agents. Lack of oxygen, for instance, affects transmission across synapses much more readily than conduction along fibers. (5) Various drugs act, like curare, exclusively upon the motor end-plate without affecting conduction in the fiber.

It is necessary to keep in mind the special physiological and histological properties of synaptic junctions and of the motor end-plate if we want to understand the origin and the background of the hypothesis of the so-called neurohumoral transmission of nerve impulses. There was no difference of opinion in respect to the view that nerve impulses along fibers are propagated by electric currents. But in contrast to this process of conduction, the transmission of impulses from neuron to

neuron or from neuron to the effector cell was assumed to be mediated by chemical substances which are released from the nerve terminal, cross the non-conducting gap between the two conducting cells, and act as a specific chemical stimulant of the next cell. These chemical mediators or neurohumoral transmitters were postulated to substitute for electric currents as propagating agents.

The idea of neurohumoral transmission was first proposed by T. R. Elliott (1905). Impressed by the similarity of the action of adrenaline and the effect of stimulation of sympathetic fibers, he proposed the hypothesis that adrenaline may be the transmitter from sympathetic nerves to their effector organs. A comparable rôle was later ascribed to acetylcholine in parasympathetic nerve endings. This ester had attracted the interest of physiologists and pharmacologists since Hunt and Taveau (1906) described the extraordinarily strong pharmacological action of this compound. Weiland (1912), in the laboratory of Magnes, observed that various isolated parts of the gastrointestinal tract release a substance into the surrounding fluid which has a stimulating effect upon a isolated intestine. On the basis of these observations, he concluded that chemical reactions must be responsible for the automatic movements of the intestine. It was shown in the same laboratory by LeHeux (1919) that a least 75% of the substance is choline. He concluded that choline may be a physiological stimulant, a kind of hormone, for the movement of the intestines. Observations of Rona and Neukirch (1912), that organic acids in very small concentrations may excite the intestine, led LeHeux to test whether or not these acids may form, with the aid of synthesizing enzymes in the intestinal wall, cholinesters, especially acetylcholine. This explanation seemed to him a good possibility in view of the strong pharmacological actions of this ester described by Hunt and Taveau and greatly extended by Dale (1914). In extremely interesting experiments, LeHeux (1921) succeeded in demonstrating that the action of some organic acids, but especially of acetic acid, must be attributed to the formation of the cholinester. He also demonstrated in these experiments the antagonistic action of atropine towards the choline esters. Otto Loewi (1921) observed that stimulation of the heart vagus of frogs led to the appearance of a substance in the perfusion fluid which acts like stimulation of the vagus when added to a solution perfusing another frog heart. He referred to this substance as "Vagusstoff." Loewi himself considered the possibility in 1926 that the Vagusstoff might be a cholinester (Loewi and Navratil,

1926). This conjecture, supported in fact by the observations of Dale and the school of Magnes, was borne out in the following years by the work of various laboratories, especially by the evidence of the natural occurrence of the ester in animal tissue (Dale and Dudley, 1929). The various developments, to which only brief allusions can be made, appeared to support the hypothesis of neurohumoral transmission. Acetylcholine was considered to be a chemical mediator at parasympathetic nerve endings, in the sense proposed by Elliott for adrenaline at sympathetic endings.

It has been known for many years, especially from the work of Boehm (1908), that striated muscle of frog reacts strongly to quaternary ammonium compounds. In 1921, Riesser and Neuschloss, using frog and toad gastrocnemius muscles, first demonstrated that exposure to Ringer solution containing acetylcholine leads to a contracture. They were impressed by the strength of the effect and its reversible nature. Only the junctional region reacted to acetylcholine, application to either the muscle or nerve fiber was without effect. The authors also described the prominent contracture of the frog rectus abdominis and the antagonism of the effect of acetylcholine by either atropine or curare. In the following decade, the behavior of frog and warm-blooded muscle to acetylcholine was investigated in many laboratories and a considerable amount of information accumulated as to the effect of acetylcholine on striated muscle. But it was not until Dale and his associates applied the technique of close intra-arterial injection that it became possible to demonstrate twitch-like responses of the muscle to acetylcholine (Brown *et al.*, 1936). Moreover, these investigators were able to demonstrate the appearance of acetylcholine in the perfusion fluid of the neuromuscular junction following stimulation (Dale *et al.*, 1936). On the basis of these observations, they proposed the hypothesis that acetylcholine might be a neurohumoral transmitter across the neuromuscular junction, i.e. that it was released from the nerve terminal and stimulated the muscle. The strong action of acetylcholine on synaptic junctions might have been considered as a purely pharmacological effect. The second type of findings, however, indicated that acetylcholine must have a physiological function in nerve activity, although the type of observations obviously was not adequate for an interpretation of the finer cellular mechanism.

Whereas the idea of the neurohumoral transmission appeared acceptable to many physiologists in the case of the autonomous nervous

system, the situation changed when the same mechanism was proposed for synapses and motor end-plates; in this case, the hypothesis encountered strong opposition by many neurophysiologists in this country (see e.g. Erlanger, 1939; Fulton, 1939; Lorente de Nó, 1938) and in Europe (Lapicque, 1936; Monnier, 1936; von Muralt, 1937, 1946; and others). There were many contradictions, admitted even by the most ardent proponents of the hypothesis, and many difficulties remained unexplained. One of the objections, raised especially by the school of Sherrington (Fulton, 1939), was the following: the electric manifestations indicate that the properties of the cell body and of the axon are fundamentally quite similar in respect to excitability. This fact makes it difficult to assume a mechanism of propagation across the synapse fundamentally different from that carrying impulses in the axon. The problem was scrutinized by Erlanger (1939) at a symposium in Toronto on the synapse. He showed that many of the so-called peculiarities of the synapse, as for instance latency, one-way transmission, temporal summation and facilitation, transmission across a nonconducting gap, and many other phenomena, may be under appropriate conditions demonstrated just as well on the axon as on the synapse. There are many differences as to quantitative aspects, but like Fulton he arrived at the conclusion that the electrical signs do not justify two fundamentally different mechanisms of propagation. In respect to the hypothesis of neurohumoral transmission, he raised the pertinent question: "If an inactive zone in the fiber of more than 1 mm. length does not prevent the propagation of nerve impulses by electric currents, is it justified to assume that the discontinuity at the synapse would interfere with such a transmission?"

The idea of neurohumoral transmission appeared unsatisfactory in many respects. On the other hand, it was necessary to find a satisfactory explanation for the important experimental facts upon which this hypothesis was based and to reconcile them with the conclusions indicated by the electrical manifestations. At this impasse it appeared imperative to approach the problem with new methods. The notions and principles applied so successfully to the elementary process of muscular contraction, as mentioned above, appeared to offer the greatest promise for obtaining information as to the elementary processes of nerve conduction and as to the rôle of acetylcholine in these events.

Investigations of the last 20 years made it indeed possible to establish the sequence of energy transformations during nerve activity, to inte-

grate acetylcholine into the intermediary pathways of the nerve cell, and to correlate a series of chemical processes with electrical manifestations. A theory has been proposed in modification and extension of the original *hypothesis of neurohumoral transmission, which tries to* integrate the available physical and chemical data and has eliminated quite a few apparently contradictory facts. Previously, it was assumed that acetylcholine is released from nerve terminal and acts upon the effector cell as transmitter. The rôle of acetylcholine was limited exclusively to the synapse. In the theory proposed by the author in 1940, the action of acetylcholine is not an inter- but an intracellular process taking place within the conducting membrane. The action of the ester is necessary for the change of permeability of the membrane. Its action on a receptor is a trigger mechanism by which the ionic concentration gradients, inactive in resting condition, become the effective source of EMF. The action of acetylcholine thus forms an integral part of the elementary process by which bioelectric potentials are generated in the axon, in the nerve terminal, and in the postsynaptic membrane, but the electric currents are the propagating agent in transmission just as well *as in conduction.*

In the following sections, the most important facts upon which this concept is based will be briefly summarized. More detailed accounts may be found in preceding reviews (Nachmansohn 1946; 1952a, b, c; 1953/54; 1955a b, c; 1957; 1959; Nachmansohn and Wilson, 1951, 1955, 1956; Wilson and Nachmansohn, 1954).

IV. ROLE OF ACETYLCHOLINE IN CONDUCTION

A. Physiologically Significant Features of Acetylcholinesterase

If one assumes that the action of acetylcholine is essential for the rapid transitory change of permeability in the conducting membrane during the passage of the impulse, then the ester must be inactivated with very great speed. The two components resulting from the hydrolysis of the ester, acetate and choline, are physiologically virtually inactive. It has, therefore, always been assumed that inactivation takes place by enzymatic hydrolysis of acetylcholine. Studies of the properties of the enzyme performing this catalysis have provided pertinent information. (For a more detailed presentation see e.g. Nachmansohn and Wilson, 1951; Nachmansohn 1955a.)

1. *Specificity*

Esterases are widely distributed in animal tissues since a great variety of esters are metabolized in living cells. The esterase which is found in conductive tissue has, however, a number of properties by which it may be distinguished from other esterases and has, therefore, been referred to as acetylcholinesterase by Augustinsson and Nachmansohn (1949a). Among the characteristic features may be mentioned the relatively high affinity for acetylcholine, the sharply defined optimum concentration, the low rate of hydrolysis of non-choline esters at this optimum concentration for acetylcholine and the decrease of activity with the increase of the acyl chain length from two to four carbon atoms. Even serum esterase, which is a cholinesterase, is in several respects different from acetylcholinesterase.

2. *Occurrence in all Conductive Tissues*

This special type of esterase is present in significant amounts in conducting tissues throughout the animal kingdom, although minor modifications of the pattern may occasionally occur, possibly by addition of undetermined amounts of other esterases which cannot be readily separated. The enzyme is present in all types of nerve fibers, motor and sensory, central and peripheral, sympathetic and parasympathetic, vertebrate and invertebrate, and in all types of muscle. It is also present in the monocellular ciliated organism, *Tetrahymena geleii* S. (Seaman, 1951). The presence of a fairly specific enzyme in a cell indicates that the substrate is metabolized there. This is particularly true in this case since, as we will see later, conductive tissues have also an enzyme which forms acetylcholine, choline acetylase. One of the results of general interest which came out from the use of isotopes is the conclusion that enzymes do not lie dormant in a cell, but are continuously active, although of course, not all the enzyme present will act continuously or work at maximal speed (Schoenheimer and Rittenberg, 1940).

The presence of this special enzyme in all conducting tissue is a prerequisite for the assumption that the substrate catalyzed is associated with the elementary process of conduction in all conducting cells; the presence of the enzyme does not, of course, indicate its rôle.

3. *Concentration and Localization*

Extensive studies on the distribution and concentration of acetyl-

cholinesterase in conductive tissue have shown that significant amounts of ester may be split per gram tissue within a millisecond, i.e. within a period of time during which the impulse passes. Fibers are capable of hydrolyzing amounts of ester ranging in general from 5 to 50 mg. per gram fresh tissue per hour. In regions where cells and synapses are located, the concentration rises to values several times as high as those found in the corresponding fiber. The amount of acetylcholine which may be split per gram nerve tissue per millisecond corresponds to about 10^{14} to 10^{15} molecules of acetylcholine. Assuming that one molecule of acetylcholine may cover an area of about 50 square A., the amount which may be metabolized per millisecond per gram would be able to cover 50–500 millions of μ^2 of neuronal surface.

Estimates of activity in relation to surface appear justified and meaningful in connection with the special localization of the enzyme. Observations of Couteaux and Nachmansohn (1940), to be discussed later, suggested the possibility that cholinesterase in axons is localized in or near the surface membrane. In experiments with the giant axon of squid, the enzyme activity was demonstrated to be localized exclusively in the sheath, whereas no activity was detected in the axoplasm (Boell and Nachmansohn, 1940; Nachmansohn and Bettina Meyerhof, 1941). Since the active membrane must be located somewhere in the sheath, this exclusive localization appears pertinent. It shows in any case that the usual way of expressing the enzyme activity in terms of substrate hydrolyzed per gram tissue is not adequate. Just as in the case of initial heat, where Hill and his associates estimated the data in reference to a layer 50 A. thick, it would be more appropriate to refer the enzyme activities measured to a small fraction of tissue rather than to the total weight. The enzyme must be and is, as we will see later, several times in excess of the amount required for activity. Part of it may be localized not within the active membrane but in surrounding layers. Moreover, the amount of active surface per gram tissue varies greatly in different types of nerve. Since thickness and extent of the layer are uncertain, it is not possible to estimate the exact concentration per unit volume. But it is important to recognize that the actual concentrations must be very much higher than those given as rates per gram whole tissue per hour; in many, if not most, cases they are probably higher by a factor of several hundred times.

4. *Time Factor*

An outstanding feature of acetylcholinesterase, pertinent to the problem of its biological function, is the high rate at which the enzyme hydrolyzes its substrate. The enzyme has not been crystallized as yet and a final determination of its turnover number has not been achieved. In its best preparations, obtained repeatedly from electric tissue of *Electrophorus electricus*, the specific activity has been about 400 millimoles ($\sim$70 to 75 grams) of ester split per milligram of protein per hour. These preparations showed one component only in electrophoretic studies and in the analytical ultracentrifugation (Rothenberg and Nachmansohn, 1947); however, there was no sharp peak during high speed centrifugation and the material was polydispersed. New studies (by C. Lawler) are at present in progress. But the data established so far indicate that acetylcholinesterase is one of the fastest enzymes known: 1 molecule of enzyme may split 1 molecule of substrate in less than one tenth of a millisecond. The high rate of hydrolysis in combination with the high concentration of the enzyme appear pertinent in relation to the proposed rôle of the ester in the elementary process of conduction. A compound postulated to be responsible for the generation of bioelectric currents by increasing the Na-conductance of the membrane should be inactivated at a speed comparable to that of the return of Na-conductance to its initial value. This is a prerequisite of crucial importance. The activity of acetylcholinesterase satisfies this postulate.

B. Inseparability of Conduction and Esterase Activity

The features of acetylcholinesterase mentioned so far show the possibility of an association of the action of acetylcholine with the elementary process of conduction; they are suggestive that the ester plays an important rôle but they do not prove it. In order to demonstrate that acetylcholine is intrinsically associated with conduction, it is necessary to test whether or not acetylcholinesterase activity is inseparable from the propagation of the impulse.

The way to test such a problem has been first shown by Lundgaard's (1930) classical observations on the effect of monoiodoacetate on muscular contraction and lactic acid formation. In spite of complete block of this chemical process, contraction still went on. By correlating several chemical and physical events, Lundsgaard demonstrated that lactic acid formation is not essential for the elementary process of contraction. If acetylcholinesterase could be completely blocked by specific in-

hibitors while conduction was still possible, this would indeed be a very serious objection to associating this enzymatic reaction with the primary event of conduction.

In 1945, it was shown (Bullock *et al.*, 1946) that, contrary to previous reports (Cantoni and Loewi, 1944), eserine does block conduction. This compound is a very powerful inhibitor of acetylcholine, the K_I being about 6×10^{-8}. This effect on conduction is readily reversible, as might have been expected in view of the reversibility of the enzyme inhibitor complex. A dramatic development in this field took place, however, in the following year. During the war, it became known that a group of compounds, alkylphosphates, are powerful and irreversible inhibitors of esterases in general as well as of acetylcholinesterase. Alkylphosphates were developed by Schrader (1952) as insecticides. Some of them are volatile, they are the so-called "nerve gases" prepared by the German army as powerful potential chemical warfare agents, the most powerful toxic agents ever developed. One of the alkylphosphates, diisopropylfluorophosphate (abbreviated DFP), was most thoroughly investigated in various laboratories of this country and abroad. It was reported in 1946 that DFP might destroy acetylcholinesterase completely without impairing conduction (Crescitelli *et al.*, 1946). Several other data were considered to be incompatible with the role of acetylcholine in the primary events of conduction (see e.g. Symposia Physico-chemical Mechanism of Nerve Activity, 1946).

Shortly afterwards, however, it was shown that the methods applied in the earlier investigations were inadequate. Extensive investigations carried out with DFP and other alkylphosphates have demonstrated the essentiality of acetylcholinesterase in a more conclusive and, as far as the quantitative aspects are concerned, more complete way than it was possible in experiments with reversible inhibitors. Although irreversible inhibitors are in many ways more favorable for demonstrating the relationship between acetylcholinesterase activity and conduction, there are many precautions necessary in the use of these compounds and the interpretation of the results. For details, the reader interested in these particular aspects is referred to the original papers and the previously quoted reviews. Only a few of the main results may be briefly discussed insofar as they are pertinent for the problem of the rôle of acetylcholine in conduction.

(1) Under no condition is it possible to dissociate cholinesterase activity from conduction. At the time when electrical activity is irrevers-

ibly blocked, about 20% of the initial esterase was found to be active in the axons tested, indicating that the enzyme is present in excess of about 5 times the minimum value required. This has been demonstrated with a great variety of different types of nerves. The most conclusive demonstration of the inseparability of electrical and chemical activity are the observations of Wilson and Cohen (1953) on intact nerve fibers of the crab, which for various reasons are particularly suitable for this type of experiment. Both electrical and enzyme activity were measured on intact fibers. This was made possible by using the tertiary analog of acetylcholine, dimethylaminoethyl acetate, as a substrate. The quaternary compound, as will be discussed later, does not penetrate into the fiber, but the tertiary does and is a good substrate. Whatever type and whatever concentration of inhibitor was used, electrical activity disappeared at 20 per cent of the initial value of enzyme activity, only the time required varied depending on type and concentration of inhibitor; as was later observed, temperature too is an important factor.

(2) Inhibition of the enzyme by DFP is even in solution a relatively slow process and has a high temperature coefficient. A strict parallelism has been shown between the rate of inactivation of esterase and that of the block of conduction as a function of time and of temperature. At 37°C., electrical and chemical activity may be irreversibly blocked in minutes; at 5 to 10°C., the process of complete irreversible inactivation of both activities may take hours (Bullock *et al.*, 1947).

(3) The inseparability of electrical and chemical processes has been demonstrated with all types of nerves, motor and sensory, cholinergic and adrenergic, vertebrate and invertebrate, and in muscle. It has been shown with the monocellular organism of *Tetrahymena geleii* S. that the ciliar movements were blocked by either eserine or DFP (Seaman and Houlihan, 1951). The question whether or not esterase activity and conduction can be dissociated has been unequivocally answered.

One main objection has been the high concentration of DFP required to produce block of conduction, namely about .005 *M* (about 1 mg DFP/ml.). This concentration was considered as an indication of a general toxic effect or of an unspecific protein effect. The outside concentration, however, does not give any indication as to the concentration at the site of action. This has been shown by observations in which it was found that, at the time of block, the concentration of DFP inside the axon, in the axoplasm, is less than 1 μg./g. We do not know

what the concentration of DFP may be at the active site. But the decisive argument is the demonstration that no electrical activity can be observed when the enzyme activity falls below 20%. This invalidates the argument of a too high concentration of inhibitor or the speculation as to an unspecific protein effect.

C. Sequence of Energy Transformations

The measurements of heat production during nerve activity and of the extra oxygen uptake by Fenn (1927), Gerard and Meyerhof (1927), and Bronk and his associates (Brink *et al.*, 1952) have given us an indication of the overall utilization of energy. The data do not indicate what chemical energy is directly associated with either activity or recovery. The major part of the extra chemical energy developed during recovery is certainly used for the restoration of the unequal ionic distribution prevailing in the resting state. The recent values of Hill and his associates mentioned above as to the initial heat made untenable the view that conduction can be explained purely by ion movements. They only emphasize the necessity to elucidate the underlying molecular, i.e. chemical events.

It was pointed out by Otto Meyerhof in 1913, in his classical article "Zur Energetik der Zellvorgaenge," that for correlating chemical reactions with a cellular function, it is necessary to know the sequence of energy transformations taking place between the primary event and oxidation which is the ultimate source of energy. In spite of all progress achieved in the knowledge of intermediary metabolism of muscle, that associated with nerve conduction remained for a long time uncertain and unsatisfactory, as was pointed out by Feng (1936). The situation started to change when the writer initiated in 1937 investigations on the electric organ of electric fish, with the aim of obtaining information about the chemical reactions underlying bioelectric currents and the rôle of acetylcholine in this process.

1. *Electric Fish*

Bioelectric potentials were described many years before Galvani. Michel Adanson (1757) compared the sensation felt in touching a *Malapterurus* to that of the discharge of a Leyden flask. John Walsh (1773) demonstrated the shock of *Torpedo*, known to Greeks and Romans, to be an electric discharge and at about the same time Williamson (1775) made corresponding observations on *Electrophorus electricus*. After

Galvani's sensational observations, biologists became interested in these fish. Galvani himself, in the last 2 years of his life, worked with *Torpedo*. During the last century, many physiologists analyzed different aspects of the electric discharge and other physiological and structural properties of the fish. For the physiologist, the most important feature of these organs is the fact that their strong bioelectric potentials are generated in the same way as those of nerve and muscle. This was recognized by physiologists of the last century and particularly stressed by DuBois-Reymond (1877), who devoted many years of his life to the study of electric fish. He emphatically expressed his conviction that analysis of the electric discharge of this fish would eventually lead to a better understanding of the electrical manifestations in nerve and muscle.

The electric organ is formed by compartments, each containing an electric plate, the electroplax, which are arranged in columns. The action potential developed by a single electroplax is about 0.14 v., which is of the same order of magnitude as that found in ordinary nerve and muscle fibers. It is only the arrangement of these plates in series as in a Voltaic pile which enables these organs to develop the high voltage. Volta recognized the analogy. Describing his pile in a memorandum read before the Royal Society in London, in 1800, he wrote he wished to give to the pile the name: "An artificial electric organ." Only one face of the electroplax is innervated. Half a century ago, Bernstein suggested that the arrangement in series may be explained by the change of potential at the innervated face only. In conformance with his ideas on conduction, he assumed a simple depolarization. Recent work with intracellular electrodes on electroplax of *Electrophorus electricus* has shown that Bernstein's hypothesis was essentially correct; however, the innervated face is not merely depolarized but reverses the charge (Altamirano *et al.*, 1953; Keynes and Martins-Ferreira, 1953). Summation of the voltages developed by the individual cells would be impossible if both faces were to reverse their potentials.

There are several species of fish provided with electric organs. The great differences of the discharge in various species do not depend on the units which show relatively small variations, but on the shape and dimensions of the organs. In the species with the most powerful electric organ known, *Electrophorus electricus* (Linnaeus), about 5000 to 6000 electroplax are arranged in series from the cephalic to the caudal end of the organ. The voltage of the discharge is on the average about 600 v. In *Torpedo marmorata* the number of elements in series does usually not

exceed 400 to 500. The discharge here is on the average 40 to 60 v.

The electric organs have phylogenetically evolved from striated muscle. In the strong electric organs, the contractile elements have, however, completely disappeared; they exist as rudiments in the plate of the weak electric organs of Rays and may be found in the embryonic tissue of *Torpedo.*

The large literature on anatomical data and electric characteristics of electric fish accumulated by intensive investigations during the last century has been summarized in an excellent review by Rosenberg (1928). Pertinent recent observations on electric characteristics carried out mostly by Fessard and Chagas and their associates and by Coates and Cox may be found in papers by Fessard (1946), Albé-Fessard (1950), Albé-Fessard *et al.* (1951), and by Cox *et al.* (1945, 1946).

Studies with isolated rows of electroplax of *Torpedo* were initiated by Auger and Fessard in the late thirties (Fessard, 1946). They suggest that the electric plates are homologous to motor end-plates. This is apparently also the case with the electroplax of Rays (Brock *et al.*, 1953; Schoffeniels, 1958a). More recently, isolated rows of electroplax of *Electrophorus electricus* were used in the laboratories of Chagas in Rio and in those of the writer. Here the electric response of the electroplax of this species is analogous to that of a muscle fiber rather than to that of an end-plate. There are two distinct types of electric response: a graded one, which resembles the end-plate potential, and an all-or-nothing spike which appears at a critical size of the prepotential and has all the characteristics of a propagated spike (Altamirano *et al.*, 1953; Keynes and Martins-Ferreira, 1953). This confirms and extends the previous observations of direct excitability of the electroplax of this species (Albé-Fessard *et al.*, 1951).

If one believes in the biochemical unity of life, a notion so greatly cherished by Pasteur and Meyerhof and which was so useful in the development of modern biochemistry, these most powerful bioelectric generators which nature has created should be a favorable material for the analysis of the basic mechanism by which bioelectric potentials in general are generated. The underlying chemical processes should be in a range more suitable for analysis and identification than those of ordinary nerve in which the smallness of the energy transformations is a formidable obstacle.

In 1937, we utilized for the first time the electric organ for the study of the metabolic relationships of acetylcholine and their connection

with the energy transformations involved in the generation of bioelectric currents. The choice of this material proved to be decisive for the later developments.

2. *Direct Proportionality between Voltage and Acetylcholinesterase Concentration*

The first observations were most striking. The organs have an extraordinarily high concentration of acetylcholinesterase: 1 kg. (wet weight) of the organ hydrolyzes 2 to 4 kg. of acetylcholine per hour (Marnay, 1937; Nachmansohn and Lederer, 1939). This is a very high activity compared with other conducting tissues, as may be seen in Table I. This high concentration appears all the more significant in view of the high water and low protein content of electric tissue: 92% of the organ is water and only 2% is protein. The ability of the organs to hydrolyze amounts of acetylcholine several times their own weight in an hour is suggestive of a close relationship to their highly specialized function, i.e. the generation of bioelectric potentials.

This assumption is further supported by the striking parallelism between the concentration of the enzyme, the voltage, and the number of plates per centimeter in *Electrophorus electricus*. The number of plates and the voltage per centimeter in this species vary considerably with the size of the specimen and moreover decreases markedly from the

TABLE I

ACETYLCHOLINESTERASE CONCENTRATION IN VARIOUS TISSUES COMPARED TO THAT OF ELECTRIC TISSUE OF ELECTROPHORUS ELECTRICUS

Tissue	Acetylcholine hydrolyzed (mg./g./hr.)
Mammalian at 37°C.	
Muscle	5–10
Nerve fibers	10–30
Brain	20–100
Frog at 23°C.	
Muscle	3–6
Nerve fibers	5–10
Brain	40–80
Electric organ of electric eel at 23°C.	2000–4000
[illegible]	0
[illegible]	0

cephalic to the caudal end of the organ. Determinations carried out on a great number of specimens of various sizes, covering a range of action potential from 0.5 to 22 v. per centimeter, have shown a direct proportionality between voltage per centimeter and enzyme concentration (Nachmansohn *et al.*, 1941, 1942, 1946a). Other enzymes tested, respiratory and glycolytic enzymes, ATPase, etc. do not show any parallelism with the voltage developed. The activity is more or less uniform in the main electric organ. In the weaker electric organ of Sachs, where the extracellular space is extremely large, the absolute values are smaller. Most of the energy yielding enzymes, being required for a great variety of functions, must be present at many places all over the cell and the density of plates per centimeter should therefore not change the activity of these enzymes expressed per gram of tissue.

In view of the evidence for the localization of cholinesterase in or near the surface, one might have suspected that the enzyme would be localized also in the surface of the electroplax. However, in view of certain structural and functional features of the surface of the individual electroplax of this species, the direct proportionality cannot be explained in simple terms of surface localization. At the anterior end of the organ, the electroplax are very thin; both innervated and noninnervated faces have very few folds. From the cephalic to the caudal end, the thickness of the cells gradually increases and becomes markedly greater; the folds at the noninnervated face become very deep, the surface is full of digitations and thus the total surface area becomes very huge, but that of the conducting surface remains unchanged. Since voltage and enzyme concentration per cell remain the same, there is no proportionality between enzyme activity and surface but there is a direct proportionality between enzyme and conducting surface. Thus, the relationship is not only structural but functional, suggesting an interdependence between the two events, i.e. chemical and electrical activity. It still would be possible to refer the relationship to localized processes instead of to voltage, if for instance all or most of the enzyme were present at the synaptic junctions. There are many thousands of synapses per cell at distances ranging from 5 to 30 μ. Assuming average distances of 15 to 20 μ, a cell with a conducting surface of 10 mm.^{2} would have about 25,000 to 50,000 synapses. Assuming 12 μ^2 per synapse, estimated on the basis of the electronmicroscope pictures of Luft (1956), the synaptic junctions would form about 3 to 6% of the total area of the conducting membrane. The enzyme concen-

tration in synaptic regions is usually higher than in the conducting membrane outside the synapse by a factor of 3 to 5, as will be discussed in a later section. Since it is well known that the surface increases at synaptic junctions, *the increase of enzyme concentration is probably* just an expression of increased surface. The enzyme located at the synapses forms apparently a small fraction of the total enzyme. The relationship between electrical and chemical activity must, therefore, be referred to the whole conducting membrane and not just to the junctions.

There are few examples in biology where direct proportionality between a physical manifestation and a specific chemical reaction has been demonstrated. The combination of the highly specialized function of electric organs in general with the unusual structural features of the organ of this particular species created a uniquely favorable material for demonstrating a functional relationship but, as always, the finding obtains its real significance only in connection with all other data.

3. *Phosphorylated Compounds as Energy Source*

The energy released in electric tissue by the hydrolysis of phosphocreatine is adequate to account for the total electric energy released by the discharge (Nachmansohn *et al.*, 1943a, b). In addition, there is energy released by the simultaneous formation of lactic acid. The amount of energy supplied by the sum of these two reactions is more than adequate for the total electric energy, but it appears likely that the major part of the energy released is not used for the immediate process of recovery but for the restoration of the ionic concentration gradient.

It was safe to assume that, as in muscle, the breakdown of ATP during nerve activity precedes that of phosphocreatine. It is today widely accepted that ATP reacts directly with the structural muscle protein in the elementary process of contraction. However, it appeared for many reasons unlikely that ATP is responsible for the change in permeability postulated in the elementary process of conduction. It would, indeed, be surprising that nature should use the same compound for two functions of so entirely different an order of magnitude in regard to energy requirements and speed involved. If, however, the action of acetylcholine were responsible for the alterations of the membrane required for the generation of the action potential, as the available evidence suggested, then these reactions should occur prior to the break-

down of ATP. The latter should be the recovery process supplying the energy for the resynthesis of acetylcholine hydrolyzed during activity.

4. *Choline Acetylase*

This last assumption proved to be correct. In 1943, an enzyme was extracted from brain and electric tissue which in cell free solution acetylates choline on addition of ATP. The enzyme was referred to as choline acetylase (Nachmansohn and Machado, 1943). This was the first demonstration that the energy of ATP may be used for biosynthesis. Since then, a great number of endergonic reactions have been demonstrated to utilize the energy of ATP. The observations opened, moreover, the way for a detailed analysis of the mechanism of acetylation in general. It was the first time that an enzymatic acetylation was obtained in a soluble system. At that time, in the early forties, the paramount importance of acetate in intermediary metabolism as a building stone of many essential cell constituents had become apparent, mainly through the application of isotope techniques.

Shortly after the discovery of choline acetylase, it was observed, in 1943, that on dialysis the enzyme rapidly loses its activity (Nachmansohn *et al.*, 1943c). This observation suggested the presence of a coenzyme in the system, and in 1945 Nachmansohn and Berman (1946) obtained a purified preparation of a coenzyme which reactivated completely a dialyzed choline acetylase solution prepared from acetone dried powder of rat brain. In view of Nachmansohn's findings, Lipmann (1945) tested the effect of ATP on the acetylation of sulfanilamide by liver extracts and confirmed that ATP provides the energy of acetylation. *Simultaneously with Nachmansohn and Berman,* he demonstrated that the sulfanilamide acetylating system requires a coenzyme. When the two findings were presented at the same meeting, it became obvious that the formation of N and O-acyl groups used a similar if not identical mechanism (Nachmansohn, 1946; Lipmann and Kaplan, 1946). Soon it became clear that the coenzyme is used in acylations in general and Lipmann referred to it as coenzyme A (CoA).

It has been established that CoA mediates a great number of key reactions in intermediary metabolism. The reactions proceed in two steps. The first step is the formation of acetyl-CoA, with acetyl adenylate as an intermediary step (Berg, 1956). In the second step, the acetyl group is transferred by specific enzyme to various receptors. Choline acetylase has been redefined as the enzyme which transfers the acetyl

group from acetyl-CoA to choline (Korey *et al.*, 1951). The importance of acetylation and the implication of these developments for general biochemistry need not be discussed here. In regard to the physiological function of choline acetylase, it is important that the enzyme is present in all conducting tissue, not only in brain and ganglia but also in axons, in different types of nerve fibers, and in muscle. The ability of conducting tissue to form acetylcholine is an obvious corollary to the postulate of its essential role in the elementary process, just as much as the presence of acetylcholinesterase and the ability of rapid removal. The distribution of choline acetylase has not been investigated as extensively as that of the esterase, but whatever conductive tissue was tested, the enzyme was found to be present. It was shown to occur in several types of motor and in purely sensory nerves, in the dorsal roots of ox and dog (Cohen, 1956). Its presence in optic nerve (Nachmansohn and Berman, 1946) was questioned by Feldberg (Feldberg and Mann, 1946). The results were, however, based on inadequate techniques; when the optimal conditions for the reaction mixture had been better established, the values of choline acetylase concentration in optic nerve were found to be even higher than in the early experiments (De Roetth, 1951). The occurrence of the enzyme was demonstrated in striated and cardiac muscle (Nachmansohn *et al.*, 1947; Cohen, 1956) of primitive invertebrates, such as annelids, flatworms, and coelenterates (Tubularia) (Persky and Gold, 1948), and in *Schistosoma mansoni* (Bueding, 1952). The enzyme was also found in other conducting tissues. It is absent in liver and kidney.

Another point of physiological interest is the rate of disappearance of choline acetylase in degenerating nerve fibers. Feldberg (1943) claimed that, in degenerating nerve fibers, the ability to synthesize acetylcholine ceases before the ability to conduct impulses. That observation was considered as an important argument against an essential rôle of acetylcholine in conduction. These experiments were performed under conditions where neither respiration nor ATP nor an other source of energy for synthesis was available. Feldberg found that 1 or 2 gamma of acetylcholine were formed per gram per hour in normal fibers. When the activity in rabbit sciatic nerve was tested under proper conditions, it was found that acetylcholine was formed at a rate of about 100 μg. per gram fresh tissue per hour (Nachmansohn *et al.*, 1946c). Two days after section of the fiber, about two-thirds of the initial concentration of choline acetylase was still present. Con-

duction ceases on the third day. Even at that time, the enzyme activity was still about one-third of the initial. When the activity was retested under still better conditions in the reaction mixture, it was found that 1.3 g. of acetylcholine were formed per gram acetone dried powder prepared from rabbit sciatic nerve. This corresponds to about 200 to 300 μg. per gram fresh tissue per hour (Cohen, 1956).

The concentrations of acetylcholinesterase are markedly higher than those of choline acetylase. This is not surprising, as has been pointed out repeatedly (Nachmansohn, 1946; Cohen, 1956). The esterase has the physiological function of removing the acetylcholine released, within a millisecond or a fraction of it, in order to restore the resting state. The function of choline acetylase is entirely different; the enzyme does not participate in the elementary process of conduction but has only to restore the reserves of acetylcholine present in some bound form and used up during activity. This may take place over a longer period of time during recovery. The activity of choline acetylase must be evaluated in reference to the rate at which the ester may be used. In the giant axon of squid, estimated on the basis of minimal cholinesterase activity required for conduction, around 10^{-14} μμM of ester may be metabolized per $cm.^2$ per impuls, possibly 2 to 4 times less. A formation of 20 to 30 μg. of acetylcholine per gram axon per hour would probably suffice to supply the ester for several hundred thousand impulses per hour.

The evidence that ATP provides the energy for acetylcholine synthesis supports the view that the action of acetylcholine and its hydrolysis precede the breakdown of ATP in the sequence of energy transformations. It thus has become possible to integrate acetylcholine into the metabolic pathways of the cell.

5. *Depolarizing (Electrogenic) Action of Acetylcholine*

The question remained, however, whether acetylcholine is itself responsible for the permeability change associated with the generation of bioelectric potentials or is preceded by another chemical reaction. If its action is the primary event, it should have an electrogenic action.

The possibility of a depolarizing action of acetylcholine has long been suspected. Dubuisson and Monnier (1934) and Cowan (1936) were the first to consider specifically such a mode of action. At that time, the end-plate potential was not yet known and techniques using intracellular microelectrodes were not yet developed. The prerequisite

that a compound has a quickly reversible, very brief, depolarizing, i.e. electrogenic, action, is a correspondingly rapid removal of the active agent. When the extraordinarily high concentration of cholinesterase in the electric organ of *Torpedo* was demonstrated in 1937, Auger and Fessard (1939) tested the effect of eserine on the action potential of the electric organ of *Torpedo marmorata* and found it markedly depressed and the duration of the descending phase considerably prolonged.

In view of their corresponding bioelectrical and biochemical findings, Fessard and Nachmansohn decided to test whether acetylcholine injected into the electric organ might produce an action potential. Such an electrogenic effect should be expected if acetylcholine is a compound responsible for the change of permeability of the membrane during activity. In experiments carried out in Arcachon in 1939, on *Torpedo marmorata*, in which Fessard and Nachmansohn were joined by Feldberg, they were able to demonstrate that injections of acetylcholine into the electric organ perfused with eserine do indeed generate electric potentials (Feldberg *et al.*, 1940). This electrogenic effect makes it difficult to assume that the action of acetylcholine is required for recovery and supports the assumption that the action of the ester is responsible for the change in permeability associated with the nerve impulse. Other compounds may, of course, produce similar effects. But acetylcholine is the only compound found in the tissue which fulfills the requirement for such an assumption and all the known facts are consistent with this conclusion.

The physiological discharge and the electrogenic effect of injected acetylcholine differ considerably in respect to the voltage and the duration of the spike. This is to be expected. Injections are a crude technique to reproduce an event which under physiological conditions takes place within milliseconds. If acetylcholine generates bioelectric potentials, it must be postulated that it is released within a membrane (or a molecular layer) about 100 A. thick, acts on some receptor at a few A. distance, and, having produced its effect, is hydrolyzed in microseconds. In such conditions, the action may be tremendously more powerful and more rapid than in experiments in which the active compounds are injected into blood vessels. The significant result of the experiment was the demonstration of the electrogenic action of acetylcholine, even if duration and strength of action were not comparable to the physiological effect. Since then, the discovery of the end-plate potential, the development of methods applying microelectrodes for

intracellular recording, and refined methods for application of acetylcholine have made it possible to confirm in many ways the depolarizing action, i.e. electrogenic action of acetylcholine, at least at the synapse. This aspect will be discussed later on.

6. *Sensory Neurons*

The sequence of energy transformations described and the specific rôle of acetylcholine will apply to all types of neurons throughout the animal kingdom. The acetylcholine system is present in all conducting tissues. It seems indeed difficult to assume that bioelectric potentials should be generated by different mechanisms in various types of neurons. It has been stressed that sensory fibers contain acetylcholinesterase and choline acetylase and that their conduction is blocked on exposure to specific inhibitors of the esterase in the same way as in motor fibers, for instance if dorsal and ventral roots are compared. Various types of sensory nerve endings and sensory receptors were shown to respond to acetylcholine and to related compounds reacting with the acetylcholine system, such as curare, decamethonium, eserine, in a way similar to that of motor nerves, etc. (Bing and Skouby, 1950; Skouby, 1951; Zotterman, 1953; Dodt *et al.*, 1953; Granit *et al.*, 1953; Buchthal, 1953). This is all the more pertinent, since in sensory receptors there are no synaptic junctions in the usual meaning of the word. Many of the sensory receptors are simple nerve endings within which the impulse s initiated, so the system must be intracellular.

D. The Elementary Process

The picture of the rôle of the acetylcholine system in the elementary process which has emerged from the data accumulated may be described as follows (Fig. 1). In the resting condition, acetylcholine (○┬) is in a bound and inactive form. It may be tentatively called the storage form. Excitation of the membrane by current or any other disturbance leads to a dissociation of the complex, and acetylcholine is released. The free ester acts upon a receptor protein (R), and this action upon the receptor is essential for the change of ionic permeability, the increase of Na-conductance, and thus for the generation of the bioelectric potential. Some facts to be discussed later suggest the possibility that the effect of acetylcholine is to produce a change in the configuration of the protein.

The complex between acetylcholine and the receptor is in a dynamic

equilibrium with the free ester and the receptor. The free ester will be susceptible to attack by acetylcholinesterase (E). The enzymatic hydrolysis of acetylcholine will permit the receptor to return to its resting condition. The barrier to the rapid Na movements is thereby re-established. Thus the action of the enzyme leads to immediate recovery and ends the cycle of the elementary process. It is the rapidity of this inactivation process which makes rapid restoration of the membrane possible and permits the nerve to respond to the next stimulus within

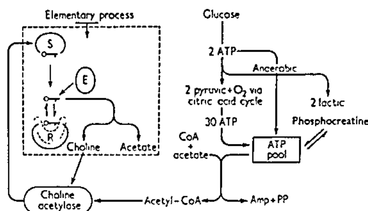


Fig. 1. Sequence of energy transformations associated with conduction and integration of the acetylcholine system into the metabolic pathways of the nerve cell. The tentative picture of the elementary process is described in the text.

a millisecond or less. All these events controlling the ion movements during activity must take place within the structural or functional barrier for these ions in rest. The further recovery leads to the resynthesis of acetylcholine in its bound form by choline acetylase and the other components of the acetylating system. Here the cyclic processes known from other cells enter the picture.

Many years ago, Otto Meyerhof raised a fundamental biological question: Living cells are able to utilize the energy of various substances such as carbohydrates, proteins, and lipids for a great variety of different and highly specific functions with about the same efficiency. How is this accomplished by the cell? The phenomenon is explained by Meyerhof in the following way. There is a specific operative substance for each specific function. This substance exists only in small quantities, but its concentration does not decrease substantially during the performance of its specific function because it is at once restored. This recovery and the maintenance of the concentration of this specific

operative substance is performed by a sequence of cyclic reactions which are partly consecutive and partly in parallel, but which are all chemically and energetically coupled. The further the cycles are removed from the specific process, the larger may be the energy supplied, and in later stages of recovery, several energy yielding processes are available and may be used either simultaneously or in turn. The specific operative substance for the elementary process of contraction is ATP, as has been proposed by Meyerhof and Lohmann and their associates and borne out by later developments. The reaction of ATP with the structural proteins, actin and myosin, appears to be the primary chemical event in muscular contraction, as has been ascertained by the work of many investigators, although here too the detailed mechanisms of this process are still far from being elucidated (for references, see e.g. Weber and Portzehl, 1954; Dubuisson, 1954; Weber, 1958).

The evidence summarized in the preceding sections has, in the opinion of the writer, established a solid basis for the assumption that acetylcholine is the specific operative substance in the conduction of nerve impulses, its action being essential for the generation of bioelectric currents. Studies on the detailed mechanism, as will be discussed more fully in a following chapter in connection with the analysis of the molecular forces acting in the acetylcholine system, are still in their initial phase. Many additional factors must be important about which virtually nothing is known. Suggestions as to the detailed mechanism of action of acetylcholine must clearly be only tentative. But a comprehensive theory of conduction cannot ignore the huge amount of data and the great variety of physical and chemical facts which have been accumulated to support the rôle proposed for acetylcholine in conduction, including the many physiologically pertinent features of acetylcholinesterase; the presence of cholinesterase and of choline acetylase in all conducting tissues throughout the animal kingdom; the evidence that acetylcholine is the first link in the chain of events associated with activity, that in the sequence of energy transformations it precedes the other reactions; the impossibility of separating these chemical from the electrical processes, etc. One type of evidence alone may be questionable, but considered altogether, these facts make the case very strong. The data appear to be as conclusive as those in favor of any biological concept or in fact of any scientific concept. All are apt to be modified and corrected.

equilibrium with the free ester and the receptor. The free ester will be susceptible to attack by acetylcholinesterase (E). The enzymatic hydrolysis of acetylcholine will permit the receptor to return to its resting condition. The barrier to the rapid Na movements is thereby re-established. Thus the action of the enzyme leads to immediate recovery and ends the cycle of the elementary process. It is the rapidity of this inactivation process which makes rapid restoration of the membrane possible and permits the nerve to respond to the next stimulus within

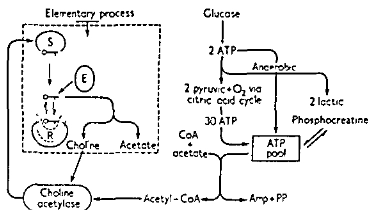
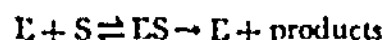


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of hydrolytic reaction in general. The process of hydrolytic enzyme action may be expressed in the usual way:



$$E + S \rightleftharpoons ES \rightarrow E + \text{products}$$

In the first phase, the enzyme (E) combines with the substrate (S) to form the enzyme-substrate complex (ES). In a second phase, the hydrolytic process takes place. The two phases will be considered separately.

1. *The Enzyme-Substrate Complex*

The positive electric charge of the normal substrate suggests that the enzyme might contain a suitably located negatively charged region which increases the enzyme activity by contributing to the attraction, orientation, and fixation of the substrate upon the enzyme surface. The existence of such a negative site has been demonstrated in various ways with the aid of competitive inhibitors and appropriate substrates (Wilson and Bergmann, 1950a; Wilson, 1952a). Dimethylethanol ammonium ion is a thirtyfold better competitive inhibitor of the esterase than the structurally similar but uncharged isoamyl alcohol. Similarly, nicotinamide, which exists at neutral pH 7 as the uncharged base, is only one-eighth as effective an inhibitor as the positively charged N-methylnicotinamide. Prostigmine and eserine are both powerful inhibitors of the enzyme. But prostigmine is a quaternary ammonium salt and its constitution is independent of pH, whereas eserine exists predominantly as the cationic conjugate acid below pH 8, and as the uncharged base at higher pH. Prostigmine inhibition does not change over a wide pH range (6–10), whereas eserine inhibition is strong in the acid range where it accepts a proton and decreases rapidly with increase of pH.

The effect of electric charge has also been demonstrated with a variety of substrates. Dimethylaminoethyl acetate ($pK = 8.3$) exists at pH below 8.3 predominantly in the cationic form, and at a higher pH in uncharged form. The hydrolytic rate measured as function of pH is high in the acid range; it decreases rapidly between pH 8 and 9, reflecting the change in electric charge.

In addition to the contributions by Coulombic forces, the methyl groups on the cationic portion of the molecule contribute to the binding by unspecific van der Waals' forces. This was shown by Wilson (1952a), using methylated competitive inhibitors of the ammonium and hydroxyethyl ammonium series, at pH 7 where all these inhibitors

V. MOLECULAR FORCES OF THE ACETYLCHOLINE SYSTEM AND THEIR RELATION TO FUNCTION

The stage of development outlined in the preceding section was reached about 1948. It then became apparent that it was necessary, as a next step for a better understanding of the precise mechanism of conduction, to study the proteins of the acetylcholine system. The situation bears some analogy to that prevailing in the late thirties in the investigations of muscular contraction, when by the observations of Engelhardt and Ljubimova (1939; Engelhardt, 1942), the interaction between myosin and ATP moved into the center of interest.

As we have seen, there are four proteins (or conjugated proteins) which act directly with acetylcholine and are tied to its function. Two of them, the two enzymes, have been isolated and are available for analysis in highly purified form in solution. The receptor may be studied on living cells. Recently, Chagas (1957) and his associates, have reported interesting and important attempts to isolate the receptor. The isolation and identification are not yet complete. Virtually nothing is known about the storage form. But fortunately, a molecule such as acetylcholine has only a limited number of possibilities to react with a protein; the molecular forces acting between the small and the macromolecules of the system must therefore be quite similar. Relatively small modifications in the surface of the protein may lead to important changes of function. Information obtained by the analysis of the molecular forces of one of these proteins may therefore offer very useful indications for a better understanding of the reactions of the ester with the other proteins. The most favorable protein for these studies is the acetylcholinesterase. This enzyme is available in a stable and highly purified form. Its activity can be easily measured by precise, simple, and rapid methods. It reacts with several substrates and a very great number of inhibitors. It is not too specific nor too unspecific. This makes it possible, by using a variety of reacting molecules, to learn a great deal about the atomic and molecular forces in the active surface.

A. Molecular Forces in the Active Surface of Acetylcholinesterase

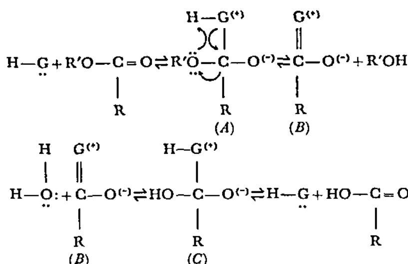
Considerable information has been obtained during the last decade, mainly by the work of I. B. Wilson and his colleagues, about the molecular forces in the active surface of the enzyme and the mechanism

and that of the basic group is about 6.5, suggesting the possibility of an imidazol ring being an active group in this process (Wilson and Bergmann, 1950b).

The enzyme-substrate complex, schematically presented in Fig. 2 is then stabilized by Coulombic and van der Waals' forces at the anionic site and by covalent bond formation between the carbonyl carbon and the basic group (G) of the esteratic site. H represents a dissociable hydrogen atom; this is not involved in the binding.

2. *The Hydrolytic Process*

Let us now turn to the mechanism of the hydrolytic process. Wilson proposed (Wilson *et al.*, 1950; Wilson and Bergmann, 1950a, b; Nachmansohn and Wilson, 1951; Wilson, 1954) the following mechanism shown below.



H symbolizes again the acidic and G̤ the basic group in the esteratic site. The pair of electrons symbolizes the electron-donating properties of the group. Acetylcholine forms the Michaelis-Menten complex (*A*). The first process is the acetylation of the enzyme with simultaneous elimination of choline. (*B*) shows the acylated enzyme depicted as an enolate ion, which is one of the resonance forms. (*C*) is an acid-enzyme complex similar to the ester-enzyme complex and leads to regenerated enzyme and acetic acid. The mechanism follows from the structure of the enzyme-substrate complex and assigns a positive rôle to the enzyme in effecting a combined acid-base attack. The acetyl enzyme reacts with water or other nucleophilic agents, such as hydroxylamine, or an alcohol, e.g. choline to yield an acid or an ester. It is possible to start

are cationic. Except for the methyl group, which becomes the fourth alkyl group, each methyl group increases the binding by a factor of 7. The exception of the fourth alkyl group will be discussed later. One can readily see that the combination of Coulombic and van der Waals' forces adds up to a very considerable high value. This explains why acetylcholine is a so much better substrate of acetylcholinesterase than, for instance, ethyl acetate.

In addition to the anionic site, there is a region in the active surface of the enzyme in close vicinity which reacts with the ester group—the esteratic site. The carbonyl group has a marked polar character. The electrophilic carbon is a site of attack for basic reagents. The importance of its electrophilic character has been tested by the strength of the inhibitory properties of a series of nicotinic acid derivatives (Bergmann

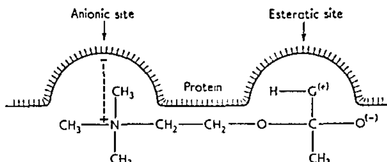


Fig. 2. Schematic presentation of interaction between the active groups of acetylcholinesterase and its substrate (The Michaelis-Menten complex).

et al., 1950a). The order of increasing electrophilic character of the carbon of the carbonyl group parallels the observed order of inhibition and suggests the formation of a covalent bond between the carbon and some basic (nucleophilic) group in the enzyme.

A further clue as to the forces in the active enzyme surface was obtained by Wilson by his analysis of pH dependence. The activity of the esterase is at a maximum between pH 8 and 9. Since the charge of acetylcholine does not vary with pH, changes in enzyme activity must therefore be attributed to changes in the protein. These changes were interpreted by Wilson in terms of the dissociation of acidic and basic groups. He postulated a number of equilibria and relations on the basis of this concept. Mathematical analysis of these relations led to an equation and predictions which were borne out by experimental results. On the basis of these studies, Wilson was able to calculate the pK's of the acidic and basic groups. The pK of the acidic group is about 9.2,

eliminating the acidic group (Wilson and Bergmann, 1950a). But whereas the physiological intermediary form, the acetylated enzyme, reacts rapidly with water (in a few microseconds), the phosphorylated enzyme reacts extremely slowly with water or not at all. If, for instance, the diethylphosphoryl enzyme is left in the refrigerator, it takes days or weeks until it becomes reactivated. DFP inactivation is not reversed by water.

If the mechanism of enzyme inhibition is based upon the attachment of the P atom to the nucleophilic group in the active enzyme surface, nucleophilic compounds should displace the phosphoryl group in the enzyme surface, whereby the nucleophilic group in the enzyme surface would become free and the enzyme would become reactivated. This proved to be the case. In 1950, Wilson obtained a relatively rapid reactivation *in vitro* with various nucleophilic compounds; the most effective was hydroxylamine (Wilson, 1951a). With a 0.7 *M* solution, a large fraction of the enzyme inhibited by tetraethylpyrophosphate was reactivated in about 300 min. This was the first demonstration that reversibility of alkylphosphate inhibition *in vitro* could be achieved in complete agreement with the mechanism proposed.

2. *An Antidote Designed on the Basis of Molecular Complementariness*

The question arose whether it was possible to accelerate the reactivation by nucleophilic agents and in this way possibly to develop an antidote against nerve gas or insecticide poisoning. The phosphorylated enzyme leaves the anionic site more or less free. Wilson reasoned that when the attacking agent is linked to a cationic nitrogen at an appropriate distance, it may greatly promote the action, just as the methylated nitrogen in acetylcholine makes this ester a several thousandfold better substrate for the enzyme than is ethyl acetate. This original idea was, in general, extraordinarily fruitful and proved to be of decisive importance in developing an efficient and powerful antidote. The first compound synthesized, primarily because it was easy to prepare, was nicotinohydroxamic acid methiodide (Wilson and Meislich, 1953; Wilson, 1955). Hydroxamic acids such as acethydroxamic acid or glycyl hydroxamic acid are poorer reactivators than hydroxylamine, but in combination with the quaternary nitrogen group located approximately at the proper distance to react with the anionic site and thereby to promote the attack of the hydroxamic acid, the reactivating power was much greater than that of hydroxylamine. It thus proved

with acids or esters, but only the undissociated acid molecules have the electrophilic carbon atom necessary for the enzyme-substrate complex. Therefore, any reaction involving carboxylic acids as substrates which is catalyzed by the enzyme must occur much more rapidly with the corresponding esters. This was found to be the case in the comparison of the enzyme catalyzed formation of hydroxamic acid and choline esters from simple esters and the corresponding acids. Reaction with the esters is about 100 times faster.

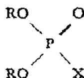
The mechanism has been confirmed in many ways (Wilson, 1951b, c). Using thiolacetic acid as substrate, Wilson found that H_2S is evolved and acetic acid is formed, as predicted by theory. The reaction is completely inhibited by prostigmine. Oxygen exchange between acids and water has been demonstrated by Rittenberg and his associates with the use of isotopic oxygen (O^{18}), again in agreement with the mechanism proposed (Sprinson and Rittenberg, 1951; Bentley and Rittenberg, 1954). The idea of an acylated enzyme being the intermediary form in the hydrolytic process, first proposed in 1950 by Wilson and substantiated by him, has since been widely accepted by many enzyme chemists and confirmed in the analysis of various enzymatic mechanisms.

Knowledge of the molecular forces acting between acetylcholine and the enzyme protein has greatly contributed to a better understanding of problems of nerve function in general. Two main lines of study will be presented as illustration.

B. Nerve Gas and Antidote

1. *Mechanism of Alkylphosphate Inhibition*

The irreversible inhibition of acetylcholinesterase by DFP and other alkylphosphates was briefly discussed before. The alkylphosphates have the general formula:



X is an acidic group. It may be F^- or Cl^- or CN^- or nitrophenol or another alkylphosphate, as for instance in tetraethylpyrophosphate. The elucidation of the mechanism of enzymic hydrolysis by Wilson led him to propose in 1949 the mechanism of enzyme inhibition by alkylphosphates. The enzyme attacks the electrophilic P atom in a S_N2 reaction,

sure lethal dose (DL_{100}) of paraoxon was injected into 20 mice. Ten of them received subsequently an injection of PAM. All of the 10 mice treated with PAM survived, while all others were dead within 40 min. (Kewitz and Wilson, 1955).

According to theory, PAM reactivates the alkylphosphate inhibited enzyme. PAM was then applied in combination with atropine, which protects the receptor. Atropine alone does not protect mice against a lethal dose of paraoxon; when combined with PAM, complete survival was obtained against ten- to fifteenfold lethal doses of paraoxon and DFP (Kewitz *et al.*, 1956).

The power of PAM to reactivate acetylcholinesterase inhibited by alkylphosphates varies depending on the nature of the alkyl group. It reactivates the enzyme inhibited by sarin and protects animals against this extremely potent nerve gas (Wilson and Sondheimer, 1957). Mice, which are particularly sensitive against sarin, are protected against 1.5-fold doses of the compound. More recently, it was reported that PAM fully protects rabbits against twentyfold lethal doses of sarin (Wills *et al.*, 1957). On the other hand, when the enzyme is inhibited by amino phosphates like tabun, no reactivation is obtained *in vitro*. A definite but small protection of the animals is still observed with PAM, but in this case it must be attributed to a direct reaction between PAM and tabun which is much faster than between PAM and either paraoxon or DFP. Thus the concentration of the active alkylphosphate in the body fluid may be reduced and some protection obtained.

A few aspects require special comments. PAM is a quaternary nitrogen derivative and practically lipid-insoluble. It does not penetrate the barrier surrounding axons (see next chapter), but it acts readily on synaptic junctions. Failure of respiration appears to be the decisive cause of death in alkylphosphate poisoning. There is general agreement as to a strong peripheral action, i.e., severe bronchial spasms, lung edema, and a paralysis of the diaphragm by the block of the motor end-plates appear all at a very early stage. However, central effects on respiratory centers seem to be additional factors, although to a varying degree depending on the type of alkylphosphate used. It thus appeared logical to use first the diaphragm for testing the effect of PAM on cholinesterase in animals exposed to severe alkylphosphate poisoning, since this material should be the most sensitive indicator of enzyme reactivation *in vivo*. Such studies were carried out by Kewitz

in principle the soundness of Wilson's argument. Moreover, whereas hydroxylamine was only able to reactivate diethylphosphoryl enzyme resulting from the inhibitory action of TEPP, nicotinohydroxamic acid methiodide reactivated for the first time enzyme inhibited by DFP, a diisopropylphosphoryl enzyme, which is much more difficult to reactivate (Wilson *et al.*, 1955). When the hydroxamic acid is in the 2-position (picolinohydroxamic acid methiodide) the compound is still more active by a factor of about 10. (Wilson and Ginsburg, 1955a). When tested on animals, some antidotal properties of these compounds were observed, but they were not impressive. Moreover, it was not certain in this case whether the protection was due essentially to the reactivation of the enzyme by the hydroxamic acid, the real aim of these studies, or due to a direct action of the alkylphosphate with the hydroxamic acid.

A much more powerful reactivator of alkylphosphate inhibited enzyme turned out to be 2-pyridine aldoxime methiodide (2-PAM) (Wilson and Ginsburg, 1955b). This compound is about a million times as effective as hydroxylamine. In order to explain the extraordinary reactivating power of this compound, Wilson and his associates studied with appropriate structures the "geography" of the enzyme surface, using trimethylphenyl ammonium ion as reference compound (Wilson and Quan, 1958; Wilson, Ginsburg and Quan, 1958). It turned out that 2-PAM has a perfect complementary conformation to the phosphorylated enzyme. The compound is a rigid and planar structure. The distance of the oxygen, the active nucleophilic atom, from the P atom of the phosphoryl group, is exactly one bond length when the cationic nitrogen is attached (by Coulombic forces) to the anionic site. If the aldoxime group is in the 4-position, the compound is still a good reactivator, although about 40 times poorer. But in the 3-position, it is inactive, in agreement with the theory derived from the geometry of the protein surface.

It may be mentioned that shortly after the description of PAM by Wilson, Childs *et al.* (1955) described a series of oximes in view of Wilson's earlier studies with hydroxamic acids; one of them was PAM. The authors recognized the improvement over the hydroxamic acids, but they did not recognize the extraordinary power of this particular oxime compared to other active oximes.

When 2-PAM was applied to mice as an antidote against alkylphosphate poisoning, the results were dramatic. In the first experiment, a

reached by PAM and PAD combined would be larger than that reached by each separately, he applied the two compounds to mice poisoned by sarin. A combination of the two compounds protected the mice markedly better against sarin, by a factor of about 50 to 100%. When a threefold lethal dose of sarin was given, all mice treated with atropine and PAM died. All those treated with the combination in which a part of the PAM was substituted by PAD survived. These developments are an illustration of how it is possible to design and modify compounds for obtaining desired biological effects.

C. Action of Acetylcholine on the Receptor

Another illustration of how the analysis of molecular forces has been helpful in the interpretation of problems of nerve function is the information obtained about the interaction between acetylcholine and receptor.

1. *Differences between Tertiary and Quaternary Nitrogen Derivatives in their Reaction with Acetylcholinesterase and Choline Acetylase*

During the analysis of the role of van der Waals' forces in the formation of the enzyme-substrate complex, a remarkable fact briefly mentioned before was observed. If one substitutes methyl groups in place of the protons of an ammonium ion, each of the first three methyl groups increase the *binding* by a factor of about 7. The fourth group is without effect. Similar results were obtained with the hydroxyethylammonium ion. The fourth alkyl (third methyl) group was without effect (Wilson, 1952a).

In striking contrast, the difference of *enzymatic activity* is extremely marked between tertiary and quaternary nitrogen derivatives; the rate of formation of acetyl enzyme by acetylcholinesterase was found by Wilson and Cabib (1956) to be about 10 times as high with acetylcholine as substrate as with its tertiary analog, dimethylaminoethyl acetate. A similar difference was observed in the activity of choline acetylase towards choline and its tertiary analog, dimethylethanolamine. The latter compound is acetylated at a rate only 8% of that of the former (Berman *et al.*, 1953; Berman-Reisberg, 1957).

How can one explain these striking differences of enzyme activity due to the presence of one extra methyl group? The binding forces, as we have seen, are not increased. The quaternary nitrogen is a saturated group and is less reactive than a tertiary nitrogen because at neutral

(1957a). Using the diaphragm of mice, he was able to show that a marked repair of the chemical lesion actually took place.

The central effects of PAM are less clear. After exposure of mice to paraoxon the esterase of the whole brain increased significantly (about 25 per cent) when the animals were treated with repeated injections of PAM (Kewitz and Nachmansohn, 1957). No such increase was obtained after exposure to DFP. However, using the whole brain is a rather crude procedure; it is possible that both alkylphosphate and PAM action is limited to certain areas. The problem requires further investigations. For details of these studies the reader is referred to the original publications (see also Kewitz, 1957b).

More than 10 years ago, the writer proposed that alkylphosphate poisoning is due to a specific chemical lesion, namely the inhibition of acetylcholinesterase (Nachmansohn and Feld, 1947). This view was at that time and for many years vigorously criticized by many investigators, mostly on the basis of the complexity of the symptoms. To attribute them to the action upon a single enzyme seemed an oversimplification. PAM, which has a complementary conformation only to acetylcholinesterase, reactivates only this enzyme with high speed. Even the closely related serum cholinesterase is reactivated only slowly and in relatively high concentrations. For chymotrypsin inhibited by alkylphosphates, PAM is a poor reactivator and 3-PAM reactivates just as well as the 2- or 4-compound. The powerful antidotal effect must, therefore, be attributed exclusively to the reactivation of acetylcholinesterase, to a repair of the specific chemical lesion. Thus, the correctness of the mode of action of nerve gas poisoning proposed by the author many years ago has been experimentally unequivocally demonstrated.

PAM is the first compound for which molecular complementarity to a special site of an enzyme has been demonstrated. It proved to have the powerful action in the animal that was the aim of the studies. One of the important aspects of this development is the demonstration that the molecular forces of the proteins of the acetylcholine system studied in the test tube are really active *in vivo*.

Some of the alkylphosphates appear to have more central effects than others. Sarin belongs to this group. As stated, PAM, is lipid-insoluble, and it is not certain whether it affects the brain. The compound does not cover the whole area which is affected by the alkylphosphates. A lipid-soluble derivative was prepared by Wilson (1958), pyridine aldoxime dodeciodide, or abbreviated PAD. Assuming that the area

the enzyme by a combination of two methods. Pieces of electric tissue containing one to three rows of electroplax were isolated. Microelectrodes were inserted into the interior of a cell; the other electrode was inserted into the tissue on the innervated outside of the same electroplax (Fig. 3). With this arrangement, the effect on the electrical activity of compounds acting on the acetylcholine system has been measured. At the same time, the esterase activity in the intact electroplax has been determined, using ethyl chloroacetate as substrate (Schleyer, 1955).

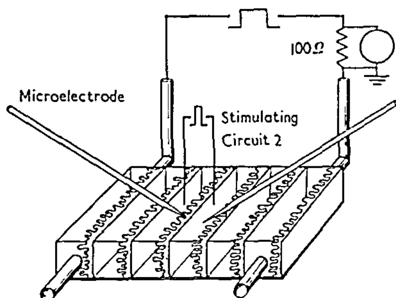


Fig. 3. Diagram of a single layer preparation of electroplax [illegible]

Neither acetylcholine nor dimethylaminoethyl acetate were found to be adequate substrates, since their penetration into the tissue is too slow. The discrepancy between "true" and "apparent" Michaelis constant, is too great to permit an evaluation of the esterase activity in the intact cell. Ethyl chloroacetate, on the other hand, is a fairly good substrate for acetylcholinesterase. There is still a discrepancy of about 30% between true and apparent Michaelis constant. However, use of this substrate permitted the demonstraion that some compounds may block conduction practically without affecting the esterase (Altamirano *et al.*, 1955; Nachmansohn, 1955a, b). Others block only if the esterase activity has fallen to low values; but in the latter case, an estimate of the actual level of residual activity is not possible, due to the inability of the substrate to reveal the total enzyme activity of the intact cell.

pH the latter is in equilibrium with a small amount of conjugated base which has a free pair of electrons. Chemical reactivity then cannot be the answer and another explanation must be found. A clue may be the tetrahedral structure of the quaternary nitrogen group. Such a structure is more or less spherical. If such a molecule is attracted to a protein surface, the fourth alkyl group will not be able to have direct contact since it is located in the direction of the solution away from the protein. The only way the protein could be simultaneously in contact with all the methyl groups would be enveloping the molecule. This implies a change of configuration of the protein during its active state in the enzymatic process.

This possibility has recently found some experimental support. Wilson and Cabib (1956) studied the enthalpies and entropies of activation, ΔH^{**} and ΔS^{**}, of the ester of ethanolamine and its methylated derivatives. Substitution of the first two protons by methyl groups did not change much the activation energies. But the extra methyl group has a very pronounced effect. The enthalpy of activation, ΔH^{**}, of the hydrolysis of acetylcholine is about 14,000 cal., as compared with about 8,000 cal. for hydrolysis of the tertiary analog. This would be less favorable for the hydrolysis of the quaternary ester. But the entropy of the activation, ΔS^{**} is extremely favorable for the quaternary compared to that of the tertiary compound. The value for the tertiary is —7 to —9 entropy units, whereas it is strongly positive with the quaternary, about +15 to +25 entropy units. This extraordinary difference in the entropy of activation produced by the presence of the extra methyl group can be explained in terms of a rearrangement of the protein molecule, i.e. a change in configuration.

2. *Evidence for the Existence of a Receptor*

Is it possible to use the information obtained with the enzymes in solution for the interaction of acetylcholine with the receptor? A change of configuration of proteins leading to a rearrangement of acidic and basic groups was proposed by Kurt H. Meyer (1937) as a possible basis of the change of permeability to ions during conduction. Does acetylcholine produce such an effect when reacting with the receptor?

The existence of a receptor has long been postulated on the basis of physiological and pharmacological observations since the time of Langley (1907). Recently, it has been possible to distinguish directly between compounds acting predominantly on the receptor and those acting on

constituent, the long postulated receptor. With compounds such as DFP, or the tertiary analog of prostigmine, the enzyme activity is at a rather low level when electrical activity stops, suggesting that in these cases, there is a strong action upon the enzyme and that electrical activity is blocked because the level of enzyme activity has fallen to a degree incompatible with electrical activity.

If the acetylcholine system is essential for the generation of the electric currents, all the protein members must be localized in close vicinity in a structural layer of about 100 A. thickness. Such structural organization would make possible a rapid and efficient action requiring extremely small amounts of the ester. But for the same reason, all compounds having structural features resembling those of acetylcholine and penetrating into this molecular layer, may react with other members of the system. The binding constants, i.e. the affinities, may vary greatly. It is known, for instance, that the K_m of acetylcholine and the dissociation constant of the carbamylcholine-enzyme complex are about 10^{-4}, whereas their dissociation constants with the receptor are about 10^{-7}. With other substances, the constants vary in the opposite direction. One might, therefore, have predicted—as has now been experimentally demonstrated—that some substances will act preferentially on the receptors, and others on the enzyme. It seems likely that in many cases both proteins are affected and that only the extent differs. Eserine, for instance, is a receptor inhibitor (Nachmansohn and Schoffeniels, 1957), but at the concentration blocking electrical activity the enzyme activity too is strongly inhibited, although not down to a critical level.

3. *Receptor Activators and Receptor Inhibitors*

The data obtained with the method described permit testing the action on the electric response of the electroplax of those compounds which act predominantly on the receptor and to determine whether the extra methyl group in quaternary compounds acting on the receptor also shows a special effect compared with that of their tertiary analogs, as was found in the studies with the enzyme proteins in solution. This proved to be the case. The compounds may be divided into two distinctly different types in respect to their effect upon electrical activity: those which block conduction without depolarization, and those which block and simultaneously depolarize the membrane. In one case, the barrier for ion movements remains closed when activity stops; in the other, it remains open. Quaternary compounds such as

Table II and III give a few data. Compounds such as carbamylcholine, decamethonium, and procaine block conduction without markedly decreasing the enzyme activity. These compounds, even at a 200-fold concentration above that producing block, still leave the enzyme activity at a high level. The blocking action cannot consequently be attributed to the effect on the esterase but to that upon another similar cell

TABLE II
RELATIONSHIP BETWEEN BLOCK OF ELECTRICAL AND CHOLINESTERASE ACTIVITY

Compound[a]	Blocking concentration (μM/ml.)	Concentration tested	Enzyme activity in per cent of initial
Carbamylcholine	0.05	0.05	99
		10.0	83
Decamethonium	0.05	0.05	86
		10.0	60
Procaine	1.0	2.0	91
		40.0	51

[a] Compounds which block response to direct stimulation at concentrations which have either a small effect or none one the esterase activity.

TABLE III
RELATIONSHIP BETWEEN BLOCK OF ELECTRICAL AND CHOLINESTERASE ACTIVITY

Compound[a]	Concentration tested (μM/ml.)	Enzyme activity in per cent of initial
Eserine	2	28
Tertiary analog of prostigmine	2	9
	4	4
DFP	1.5	4

[a] Compounds which block response to direct stimulation with a parallel inhibition of esterase activity. All three compounds block at concentrations of about 2 μM/ml.

but produces a simultaneous change. This was postulated and lucidly explained by Clark (1937) some 20 years ago. We have, therefore, introduced the distinction between receptor activators which effect this change and receptor inhibitors which combine with the receptor but do not produce a change. They latter apparently block the access of acetylcholine to the active surface. These two different types of inter-

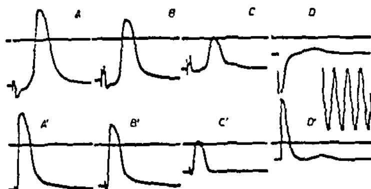


FIG. 5. Effect of prostigmine. All arrangements as in Fig. 4. A to D, neural, A′ to D′, direct stimulation; A,A′, control. B, B,′, 1 min.; C,C′, 2 min.; D,D′, 9 min.; after addition of 2.5 mg./ml. of prostigmine. At the block of conduction, resting potential again strongly decreased.

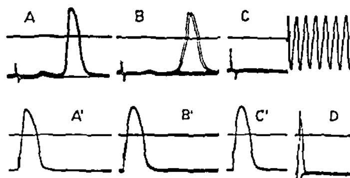


FIG. 6. Effect of the tertiary analog of prostigmine. A to C, neural; A′ to D′, direct stimulation; A,A′, control. B.B′, 2 min.; C,C′, 7½ min.; D′, 176 min. after addition of compound (1 mg./ml.). Potential difference between inside and outside remains the same. 124 min. before the last recording, 10 μg./ml. of carbamylcholine were added, but had no effect.

action are analogous to those in enzyme chemistry where we distinguish between enzyme substrates and inhibitors.

If one accepts the idea that the molecular forces acting between acetylcholine and the proteins associated with its function are more or less similar, one may visualize that the receptor in the active state undergoes a change in configuration comparable to that suggested to

acetylcholine, carbamylcholine, decamethonium, etc., belong to the latter. Figure 4 shows the effect of carbamylcholine and is typical for compounds blocking the response with *simultaneous depolarization.* On the other hand, tertiary acetylcholine analogs such as procaine block the activity but do not depolarize. An interesting example of how the presence of the extra methyl group profoundly changes the effect

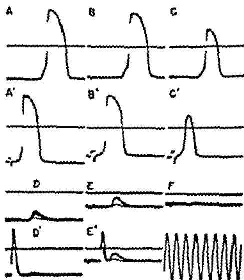


FIG. 4. Effect of carbamylcholine on the response of the electroplax of *Electrophorus electricus* to stimulation. Experimental arrangement in this and the two next figures: the tip of one microelectrode is fixed on the outside of the electroplax as close as possible to the innervated membrane; the other electrode is inserted through the noninnervated face and fixed just opposite the first electrode. The upper horizontal line appearing in the cathode ray oscilloscope corresponds to the potential difference between the two electrodes as long as both are outside the cell, i.e. the difference is zero. As soon as the second electrode is inserted, the lower line appears. The distance between the two lines indicates the potential difference in rest between the inside and outside, usually about 80 mv. A to F, neural; A' to E', direct stimulation; A,A' control. The records were taken in the following intervals after addition of 10 µg./ml. of carbamylcholine · B,B', 6 min.; C,C', 18 min.; D,D', 23 min.; E,E', 35 min.; F, 53 min. Stimulation. 25/sec. Calibration 1000 cycles and 100 mv. As the records show, not only is the propagated spike blocked, but the potential difference in rest is decreased.

on electrical activity is the difference between the effect of prostigmine (Fig. 5) and that of its tertiary analog (Fig. 6), although in this case there is an additional charge effect (Altamirano *et al.*, 1955; Nachmansohn, 1955a, b, c).

If one associates the transient change of permeability in conducting tissues with the reaction between acetylcholine and the receptor, one must assume that acetylcholine not only combines with the receptor

but produces a simultaneous change. This was postulated and lucidly explained by Clark (1937) some 20 years ago. We have, therefore, introduced the distinction between receptor activators which effect this change and receptor inhibitors which combine with the receptor but do not produce a change. They latter apparently block the access of acetylcholine to the active surface. These two different types of inter-

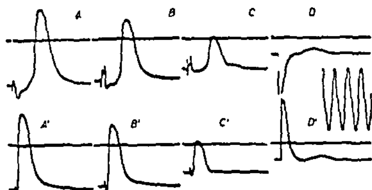


FIG. 5. Effect of prostigmine. All arrangements as in Fig. 4. A to D, neural, A′ to D′, direct stimulation; A,A′, control. B, B,′, 1 min.; C,C′, 2 min.; D,D′, 9 min.; after addition of 2.5 mg./ml. of prostigmine. At the block of conduction, resting potential again strongly decreased.

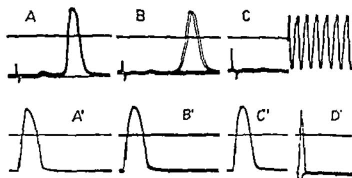


FIG. 6. Effect of the tertiary analog of prostigmine. A to C, neural; A′ to D′, direct stimulation; A,A′, control. B.B′, 2 min.; C,C′, 7½ min.; D′, 176 min. after addition of compound (1 mg./ml.). Potential difference between inside and outside remains the same. 124 min. before the last recording, 10 μg./ml. of carbamylcholine were added, but had no effect.

action are analogous to those in enzyme chemistry where we distinguish between enzyme substrates and inhibitors.

If one accepts the idea that the molecular forces acting between acetylcholine and the proteins associated with its function are more or less similar, one may visualize that the receptor in the active state undergoes a change in configuration comparable to that suggested to

occur in the enzyme during activity. Such an action may well be associated with the change of permeability taking place in the active membrane. One possible picture would be that positively charged amino groups would prevent the passage of Na^+. A small, even very limited, but strategically located change in a long protein chain may remove the positive charge by a few A. and thereby open the passage. Folding or unfolding of a small section of helical or non-helical part of a chain may be enough for such a removal and thus act as a trigger process. The data available indicate that the action of one molecule of acetylcholine may permit the passage of 500 to 1000 Na^+ ions. This is a relationship of an order of magnitude consistent with that which one would expect for a trigger process in which the controlling energy required must be small.

Since a receptor inhibitor must react with the same receptor site as a receptor activator, it follows that if an inhibitor is applied first, it should prevent the activation of the receptor by the subsequent addition of an activator. It has been found that depolarization by the receptor activator carbamylcholine is antagonized by either procaine or D-tubocurarine or the tertiary analog of prostigmine. Since the receptor inhibitors and activators are competitive, the effect of one should be overcome by increased concentrations of the other and this proved indeed to be the case (Schoffeniels and Nachmansohn, 1957).

The features required for a molecule to be a receptor activator are still poorly understood. If we refer again to the knowledge acquired by enzyme chemistry, we know that enzyme inhibitors require much less specificity than substrates. If we compare, for instance, the rates of hydrolysis of acetyl- and propionylcholine by acetylcholinesterase, there is either little difference or none. Butyrylcholine, on the other hand, is very poorly hydrolyzed, although it is bound twice as strongly to the enzyme as is acetylcholine. No information is available as to why in this case addition of one methyl group should so effectively depress the enzyme activity.

The methylated quaternary group seems to be an important factor in promoting activation. The carbonyl group is quite evidently another factor. Moreover, as in the case of the two enzymes of the system where the tertiary analogs are much poorer substrates by a factor of 10 and 15, respectively, dimethylaminoethyl acetate is also a receptor activator, but only at higher concentrations; in the case of the electroplax, the effective concentration is about 20 times higher, while in

some muscles it is much higher than that of acetylcholine, by a factor of 100 or so, as has long been known. Of course, the nitrogen of this tertiary analog is cationic, since at the usual pH it accepts a proton. It is also well known that nicotine, although it has no quaternary nitrogen, has a strong depolarizing action, i.e. is a powerful activator. On the other hand, D-tubocurarine, although containing two quaternary nitrogens, is a receptor inhibitor. Here the reason is not difficult to understand. The molecule is rather large, it contains six rings and the two nitrogens are members of heterocyclic rings. The compounds with curare-like action will be discussed later in more detail.

The similarity of receptor and enzyme offers important physiological and pharmacological problems. Of special interest are certain observations with alkylphosphates. In the earlier phase of the development, two findings in addition to those described before were considered as serious difficulties for the theory proposed by the writer. Block of conduction produced by DFP is for quite some time readily reversible. Since the cholinesterase inhibition is irreversible, this phenomenon appeared puzzling and was considered as evidence for a general toxic effect. Only the reaction with the enzyme leads to the irreversible phosphorylated protein form, during elimination of the acidic group attached to the P atom. If we assume, as appears likely, that DFP affects also the receptor as an inhibitor, one should expect a reversible block of conduction as long as the enzyme has not reached a critically low level. Just in the case of DFP, this takes quite some time, since the inactivation of the enzyme is a relatively slow process. The experimental findings are in agreement with expectation. The second difficulty for the theory arose from the observation that conduction is for a certain period of time blocked without depolarization. This was reported by Tomas and associates (1947), with frog sciatic nerve. The finding has been confirmed in our laboratory with the electroplax (Altamirano *et al.*, 1955). Since DFP has no nitrogen group, its combination with the receptor would not be expected to effect an activation. Depolarization observed in both instances in a later stage may be the result of acetylcholine accumulation, due to block of its hydrolysis.

4. *Isolated Single Electroplax Preparation*

The results discussed so far were obtained with isolated pieces of electric tissue consisting of one to three rows of electroplax. Studies with this multicellular preparation offered considerable difficulties and

contradictions. The effects obtained with acetylcholine or analogous chemical structures were all irreversible; in addition, the large extracellular spaces and extracellular structural barriers made interpretation in many instances difficult.

In the last 2 years, Schoffeniels (1957a) developed a method in which he used a single isolated electroplax. Preparations of single cellular units have frequently been of paramount importance in studies aimed at the understanding of cellular function. The new method has contributed greatly to the resolution of previous difficulties; it has offered much new and valuable information and is a promising tool for further studies. The method is patterned after that developed by Ussing and his associates with the frog skin for studies of ion transport (see e.g. Ussing, 1949, 1954). A single electroplax is dissected and kept between a nylon sheet with a window adjusted to the dimensions of the cell and a grid consisting of nylon threads; it is placed between two chambers in such a way that the cell separates two pools of fluid. The innervated membrane of the electroplax has a rectangular shape and is therefore uniquely suitable for the study of ion movements between the two chambers across the membranes. One face is innervated and has a conducting membrane, while the other is not innervated. It is possible with radioactive material and appropriate arrangement to follow the rates of flux across the two types of membranes separately. The preparation is superior to those previously used. It eliminates many disturbing factors in the study of physical and chemical effects on ion flux and on electrical manifestations. With the new preparation, the effects with acetylcholine and analogous compounds were for the first time produced in a reversible way and are therefore more closely related to the physiological event (Schoffeniels and Nachmansohn, 1957). The data obtained with the new preparation confirmed the idea of two categories of compounds affecting electrical activity, receptor inhibitors and receptor activators. They revealed a new and interesting feature distinguishing these two types. The effect of the inhibitors may be completely reversed even after relatively prolonged time of exposure. In contrast, the effect of activators cannot be reversed completely and after a certain time the electric activity once again decreases. This appears not surprising and may be explained in the following way. Keeping the barrier open to ion movements may result more readily in irreversible damage than keeping it closed. Moreover, the depolarization reflects structural changes which take place for a rather long

period of time, compared with the millisecond of the physiological process. This may lead to irreversible changes of the receptor protein or of other secondary factors.

An important new fact was the demonstration that the quaternary nitrogen derivates act exclusively upon the synaptic junction. Curare in low concentrations, about 10^{-4} *M* or less, blocked the response to indirect but not to direct stimulation. Even in very high concentrations, the propagated spike is not affected. In presence of curare in low concentrations, acetylcholine, prostigmine, carbamylcholine, and other receptor activators have no effect on the response to direct stimulation, even when the compounds were used in higher concentrations than those which were effective in absence of curare. In previous experiments, the depolarization of the membrane was erroneously interpreted to be a direct action of quaternary nitrogen derivatives on both synaptic and conducting membranes. Thus, the conducting membrane of the electroplax is just as impervious to quaternary ammonium ions as are the axons or the muscle fiber, as will be seen from the discussion in the next section. Tertiary analogs, on the other hand, known to react specifically with the acetylcholine system, have the same blocking effect as that shown with other conducting membranes. The question arises how the depolarization of the whole membrane may be explained if the quaternary compounds act only upon the synaptic junction. The innervated membrane has not one junction, as have most muscles, but as discussed before it has a very large number of them. Each time the potential is changed across a small area of cell membrane, there is a gradient of potential between the depolarized area and the surrounding membrane. If the depolarized areas are as numerous as in the electroplax, their depolarization will short circuit the conducting membrane and depolarize it.

By still further improvements in dissecting techniques in which most of the extracellular space was removed, Schoffeniels (1957b) was able to use the single isolated electroplax preparation for ion flux determination. With the aid of radioactive sodium, he was able to determine the intracellular sodium concentration in the usual way. It was found to vary between 4.5 and 23 μmoles per milliliter of intracellular water. The experiments on the flux rates of Na^+ and K^+ under different conditions across innervated and noninnervated membrane are at present in progress. The comparison of two functionally different membranes of the same cell may offer clues about special properties of conducting

membranes. In view of the great number of synaptic junctions, the preparation offers the possibility of distinghuishing to a certain degree between ion flux across the conducting membrane and that across the junctions.

The studies described above have offered experimental evidence for the existence of a receptor by observations on intact cells. During the last few years Chagas and his associates (see Chagas, 1959) have made the first and interesting attempt to isolate the receptor from extracts of electric tissue by injections of radioactive curare-like compounds. However, the component which was finally isolated turned out to be a polysaccharide; no identification with the receptor was tried. Recently, Ehrenpreis (1959a, b) using different procedures succeeded in isolating and identifying the acetylcholine receptor protein. He separated the proteins of electric tissue by ammonium sulfate fractionation and tested the binding power of the various proteins to compounds supposed to react with the receptor by equilibrium dialysis, under strict control of ionic strength and pH. He isolated a protein which has a strong and specific binding to acetylcholine, various choline esters, curare and other mono- and diquaternary nitrogen derivatives. The identification of this protein with the receptor has been achieved by comparing the binding power of the different compounds with their effectiveness on the electrical activity of the isolated electroplax. A striking parallelism was obtained.

5. *Effect of Lipid-Soluble Quaternary Ammonium Ions on Conduction*

One of the aims of designing PAD, the lipid-soluble analog of PAM, was to test the possibility of a reversal of the blocking action of DFP in the axon; it appeared interesting to try to ascertain whether conduction would reappear. When however, the compound was applied to axons, it was found that PAD rapidly blocks conduction in relatively low concentrations (Schoffeniels *et al.*, 1958). This was the first demonstration that a quaternary nitrogen derivative, when made lipid-soluble, blocks conduction. Other quaternary nitrogen derivatives were therefore prepared, including a lipid-soluble analog of acetylcholine, since this ester, as will be discussed more fully in the next chapter, does not affect conduction. When one methyl group on the nitrogen is replaced by a dodecyl group, the compound becomes very lipid-soluble. It has been referred to as noracetylcholine 12. This lipid-soluble analog of acetylcholine proved to be a potent blocking agent when applied to the

isolated single electroplax. When the effects of these lipid-soluble quaternary ammonium ions are tested with intracellular electrodes on the latter preparation, depolarization is obtained even after complete curarization, consistent with the assumption that these analogs activate the acetylcholine receptor and mimic the physiological change of permeability. In our first observations, all our records clearly showed overshoot; later no such overshoot was obtained. We are at a loss to explain the earlier findings.

The lipid-soluble quaternary nitrogen derivatives produce muscular contractions both of frog rectus and sartorius muscle. In contrast to the lipid-soluble nitrogen derivatives, this action is not blocked by high concentrations of curare (Hinterbuchner *et al.*, 1958; Hinterbuchner and Wilson, 1958a, b). Therefore, the compounds must act on the conducting membrane directly. The effects of noracetylcholine 12 on the rectus are reversible and may be many times repeated. The effects on the muscle emphasize the significance of the action of the lipid-soluble analog of acetylcholine, because they are able to reproduce the biological action postulated for the physiological rôle of the ester.

Effects similar to those obtained with noracetylcholine 12 on axons of crab and lobster and on the electroplax of *Electrophorus electricus* were recently obtained by R. Staempfli (1958) in experiments on Ranvier nodes of frog sciatic nerve. In 10^{-3} *M* concentration, the compound produced a complete breakdown of the membrane potential. In concentrations of 3×10^{-4} *M* (10 μg. per milliliter) the compound increased the amplitude and duration of the action potential. The effects on the membrane take place within seconds, with a rather high speed, only slightly less than those of K^+. The action is reversible if the compound is applied in low concentrations; but even in concentrations up to 10^{-3} *M* it is at least partly reversible.

Similar and even slightly more potent effects on the conducting membrane of the desheathed frog tibialis nerve were obtained with PAD (Dettbarn, 1959a). This quaternary ammonium ion has a marked depolarizing action at 10^{-4} *M* and a small but definite effect at 10^{-5} *M* (equivalent to 2 μg. of acetylcholine). In still unpublished experiments on Ranvier nodes of single fibers of frog sciatic nerve Staempfli and Dettbarn found depolarizing effects with PAD in 10^{-6} *M* concentrations. The electrical activity of this preparation is affected by PAD in 10^{-7} *M* concentration or less. (Dettbarn, unpublished experiments).

The effects observed differ from the physiological events. It cannot

be expected that these compounds applied externally mimic exactly the action of the ester released within the conducting membrane and removed within a fraction of a millisecond, especially since PAD is not hydrolyzed at all and noracetylcholine 12 very poorly.

For the interpretation of these effects, the question is crucial whether or not they are produced by a specific action upon the acetylcholine system, postulated to be physiologically the cell constituent whose reaction with acetylcholine is essential for the permeability change. The presence of a powerful acetylcholine system in all conducting tissues in or near the surface suggests this mode of action. But if it were possible to demonstrate convincingly that this is the basis for the depolarizing action of lipid-soluble quaternary ammonium ions, it would be a rather conclusive evidence that the slightly modified molecule has the intrinsic ability to affect the change attributed to the naturally occurring ester.

Evidence in this direction has now been obtained (Dettbarn, 1959a; Dettbarn *et al.*, 1958). If PAD depolarizes the membrane by reacting with the acetylcholine receptor, i.e. if it is a receptor activator, a receptor inhibitor should, by competition, protect the active surface of the protein against the quaternary ammonium ion. This is indeed the case. Eserine has, as is well known, a high affinity for the acetylcholine system. It is a strong inhibitor of the enzyme, the K_I is 6×10^{-8} (Augustinsson and Nachmansohn, 1949b); this is an affinity of the same order of magnitude as that which some of the best bound coenzymes have to their enzymes. Eserine is also a receptor inhibitor (Schoffeniels and Nachmansohn, 1957). When eserine, in 7×10^{-3} *M* concentrations, is added to the desheathed frog sciatic nerve, addition of PAD in 10^{-4} *M* concentration has absolutely no effect. Lower concentrations of eserine strongly counteract the effect of PAD, but do not abolish it. Higher concentrations of PAD may again overcome the protective action of eserine. The antagonism between the two compounds is typical for a competitive action. The concentration of eserine must be higher since only the free base is lipid-soluble and penetrates into the cell. The pK of eserine is 8.2; at neutral pH, only a small fraction of eserine therefore exists as the free base.

Still more recent observations of Dettbarn (1959b) indicate that these lipid-soluble quaternary ammonium ions specifically increase the permeability to Na; that to K is either not affected or may be even decreased depending on the concentration of the compounds.

The action of lipid-soluble quaternary ammonium ions on the conducting membrane is a new development; it offers many possibilities for a new approach to the study of the chemical mechanisms underlying nerve activity. Further investigations will be necessary for exploring the various aspects of modification of molecular structure in relation to biological function. But the fact that these analogs of acetylcholine have such a powerful, fast, and reversible depolarizing action on nerve and muscle fibers as postulated by theory, and the evidence that they act on the acetylcholine receptor, provide a new strong support for the proposed rôle of acetylcholine in conduction, especially if considered in connection with the huge amount of physical and chemical data accumulated in the last two decades.

6. *The Complex Nature of the Permeability Change*

The study of the molecular forces acting in the acetylcholine system has been valuable for the understanding of some aspects of nerve function; we have been enabled to manipulate some physical processes by specific chemical agents, acting on well defined proteins, in a way which may be at least partly predicted from our knowledge of the proteins in solution. However, whereas the essential rôle of the system in the permeability change is well supported by a great variety of chemical and biochemical facts, the precise mechanism of its action remains unknown. Here our knowledge and our ideas are in an initial stage and mostly conjectures. It is difficult to assume that any single system should be the only factor regulating a biological function. The acetylcholine system is part of a complex membrane formed by many additional constituents. Virtually nothing is known about the interaction between the acetylcholine system and other chemical constituents of such as phospholipids, steroids, amino acids, other metabolites, ions such as calcium and magnesium, etc.

Since early in the century much thought and many efforts have been devoted to the physicochemical properties of cellular and particularly conducting membranes. Progress was slow until recently, but advances in two fields are now changing the picture. Electron microscope and X-ray diffraction studies have brought new information concerning the ultrastructure of cells and their membranes. A special "plasma" membrane has been observed with the electron microscope, possibly identical with the conducting membrane. Some information has been obtained about the possible arrangement of proteins and lipids

within the membrane, although no specific identification has been possible as yet (see e.g. Robertson, 1958).

The second line of advance has been made possible by the availability of radioactive and stable isotopes. New information about functional aspects of cell membranes and barriers surrounding the cell has been obtained during the last decade. A huge volume of work has been devoted to the problems of ion transport across membranes. Several recent books and symposia deal with this subject (see e.g. Clarke and Nachmansohn, 1954; Symp. Soc. Exp. Biol., 1954; Discussions Faraday Soc., 1956; Harris, 1956; Murphy, 1957; Colloque. Saclay, 1958). There is general agreement, that for "active" transport, i.e., movements against the concentration gradients, metabolic energy is required. During nerve activity Na and K move from higher to lower concentrations. Here only a trigger action is required for changing rapidly and reversibly the conductance. This is a process specific for the conducting cells and here the existence of a specific system appears *a priori* likely. The acetylcholine system has the properties required for the specific function. But the action of acetylcholine has clearly nothing to do with the Na or K pump, an erroneous interpretation sometimes encountered in the literature. About the precise chemical mechanism underlying active transport virtually nothing is known except that some observations, especially those of Whittam (1958) on red blood cells, have provided good evidence for the assumption of ATP being the immediate source of energy.

One of the most challenging problems is that of the ability of the membrane to distinguish between Na and K. Considerable differences of physical factors may occur between certain salts of sodium and potassium. Some salts of polyphosphates show specificity with respect to the degree of ionization (Von Wazer and Campanella, 1950). Organic compounds have been described by Schwarzenbach and associates (1945, 1946) which form complexes with Na; they have a weak affinity for Na^+ but not for K^+. Lamm and Malmgren (1940) reported that the polymers of metaphosphoric acid have a special affinity for Na^+. Polyphosphates, including nucleic acids and phospholipidic acids, have been proposed as carriers, the latter exchanging a cation with choline. They may also play a rôle in passive movements (Vogt, 1957). Although some cases suggest a distinction between Na^+ and K^+ carrier, in other cases the observations reported suggest that the same carrier may be used in a transport exchanging K^+ and Na^+.

Many of these views, although derived from careful and extensive investigations, are still a matter of conjecture. But the existence of such chemical forces and their rôle in ion movements in combination with structural arrangements can hardly be questioned. The acetylcholine system must act within this complex framework, but at present there is no indication whatsoever how these interactions may take place. This, of course cannot be construed as argument against an essential rôle of the system.

Another point may be briefly discussed. Excitation and inhibition of the conducting process seem frequently to be a kind of mirror image although there are differences in time relations. Acetylcholine may produce activation by depolarization and inhibition by hyperpolarization. Vagus stimulation produces hyperpolarization and has a inhibitory action. The rôle of acetylcholine in this process has long been postulated and has been recently confirmed with intracellular electrodes (Burgen and Terroux, 1953; del Castillo and Katz, 1955a). Hyperpolarization in the axon by anodal currents may be produced in a related way. Such a dual function of a system is in no way unusual. It has been demonstrated for the ATP actomyosin interaction by Bozler (1951) and by H. H. Weber and his associates (see e.g., Weber, 1958); ATP controls both contraction and relaxation. A factor discovered by Marsh (1951) and Bendall (1951) and referred to as Marsh-Bendall factor, probably a protein, appears to have an essential function in directing the effect one way or the other and this factor in turn is controlled by magnesium and calcium ions. The factor lowers the optimal concentration of ATP necessary to produce maximal tension. Thus, a concentration of ATP which may produce maximal tension in the absence of the factor, becomes inhibitory in its presence. This situation is an excellent demonstration of how even in a relatively simplified biological system, the same elementary reaction may behave differently and be directed in a seemingly opposite way by additional factors. Exactly how the two opposite effects are produced by ATP is not yet understood. The work of Huxley and Hanson (1955, 1957; Hanson and Huxley, 1957) suggests that during contraction, the actin and myosin filaments slide past each other. In this process, the energy of ATP is essential, although the mode of action is obscure. A tentative picture has been presented of how the nucleotide may act; but this is at present still a working hypothesis (Weber, 1958). Excitatory and inhibitory effects which are dependent

on concentration are known for many compounds. Activation and inhibition of an enzyme activity are sometimes observed even in an isolated enzyme system as a function of substrate concentration, resulting in the so-called bell-shaped curve of activity substrate concentration relationship; even here the process is not yet well understood, although only two components are reacting in this case. At present, we can only say that the ability of acetylcholine to act in opposite ways, i.e. to produce either depolarization or hyperpolarization, has been experimentally demonstrated. It is one step to find that a biochemical system has the prerequisites to be associated with an elementary cellular function, be it ATP actomyosin with contraction or the acetylcholine system with conduction. It requires much more knowledge to describe the exact mode of action of the system and to be able to explain modifications of physical manifestations.

VI. NEUROMUSCULAR TRANSMISSION

Having summarized in the preceding sections the evidence for the essential rôle of acetylcholine in the elementary processes of conduction, we now turn to the problem of the transmission of nerve impulses across synapses in general and especially across the neuromuscular junction. The neuromuscular junction has been the subject of a considerable number of studies and has played a special rôle in the early development of the hypothesis of neurohumoral transmission. Several questions arise. Are there differences between conduction and transmission, and if so, in what respect do they differ? Does our present knowledge justify the assumption of a special chemical mechanism of transmission across synaptic junctions? More specifically, is there good evidence that the mode of action of acetylcholine, the rôle of the acetylcholine system at junctions, is distinctly different from that in the axon?

A. Re-evaluation of the Basis of the Original Neurohumoral Transmitter Theory

1. *Permeability Barrier around the Axon*

Let us first consider the two main lines of observations upon which the hypothesis of neurohumoral transmission was originally based. (1) Acetylcholine has an extremely powerful action on the junction (Brown *et al.*, 1936). Applied to the fiber, it is inactive, even in high concentrations. (2) If nerve fibers are stimulated, acetylcholine appears in the perfusion fluid of the corresponding junction although only in presence

of eserine, whereas no acetylcholine leaks from the fiber into the surrounding fluid (Dale *et al.*, 1936; Dale, 1937). These facts are interesting and have not been questioned. But what is their meaning? Do these findings really indicate that the physiological function of acetylcholine is limited to synaptic junctions and acts in the manner proposed in the hypothesis of neurohumoral transmission, or is there another interpretation possible or preferable in the light of later developments?

The lack of response of nerve and muscle fiber to externally applied acetylcholine has found its explanation in the evidence that a permeability barrier impervious for acetylcholine surrounds the axon and the muscle fiber. Acetylcholine is a methylated ammonium ion and, like other methylated quaternary nitrogen derivatives, it is lipid-insoluble. One could, therefore, expect that it would not penetrate into the interior of cells. Even in absence of myelin, a lipid membrane may render the entrance very difficult or impossible.

The presence of a permeability barrier was demonstrated in several ways (Bullock *et al.*, 1946). As mentioned before, eserine blocks conduction. Eserine is a tertiary nitrogen derivative and at neutral pH, it is partly uncharged. It penetrates into the exterior. Its presence has been demonstrated in the axoplasm of the giant axon of squid, after exposure. In contrast, prostigmine is a quaternary nitrogen derivative; the nitrogen is cationic and surrounded by three methyl groups. *In vitro*, it is a powerful inhibitor of acetylcholinesterase; the K_I is about the same as that of eserine. But if nerve fibers, even so-called unmyelinated fibers, as e.g. crab nerve fibers or the giant axon or fin nerve of squid, are exposed to prostigmine in concentrations of 0.01 *M*, conduction is completely unaffected. Even after long exposure of the axon to prostigmine, the compound is not found in the axoplasm. In the experiments with intact crab nerve fibers mentioned above, in which cholinesterase was determined on intact nerves with dimethylaminoethyl acetate as substrate, prostigmine even in high concentration was found to block only about half of the enzyme, a fraction which apparently is readily accessible even to lipid-soluble compounds and is therefore referred to as "external" enzyme. But it did not depress the remaining enzyme activity, the "internal" enzyme, to the critical 20% level and consequently did not affect conduction. When, however, eserine or the tertiary analog of prostigmine was used, both the "internal" enzyme activity and the electrical activity were blocked, although the inhibitory

power of the tertiary compound is only 1% of that of prostigmine (Wilson and Cohen, 1953).

The chemical structure of prostigmine is very closely related to that of acetylcholine; it is a typical competitive inhibitor of acetylcholinesterase. Nevertheless, it was questioned whether the barrier shown for prostigmine applied similarly to acetylcholine. Acetylcholine was therefore labeled with isotopic nitrogen (N^{15}). Giant axons of squid were exposed for 1 hour to 20 g. per liter of labeled acetylcholine. When the axoplasm was tested for excess N^{15}, the amount corresponded to about 1 µg. of acetylcholine per gram of tissue; only traces had penetrated the interior. In contrast, when the axon was exposed to tertiary amine labeled with N^{15}, equilibrium between inside and outside concentration was reached within an hour (Rothenberg *et al.*, 1948). It appears not too likely that a biological permeability barrier is absolute, but a very low degree of permeability may slow down the entrance to a rate which renders a compound biologically inactive.

In the experiments demonstrating a permeability barrier, unmyelinated fibers were used. In frog sciatic nerve the axons are surrounded by large amounts of lipid and connective tissue in addition to their thick myelin layer. Thus, the absence of any effect of acetylcholine in these experiments upon conduction must be expected. The inability of acetylcholine to act upon the axon, once permeability difficulties are recognized, is no objection to the theory attributing an essential rôle to acetylcholine in the primary process of conduction.

Although it is certain that myelin offers a nearly unsurmountable barrier, it should not be considered as the only responsible factor. It has long been known that certain quaternary compounds and specifically acetylcholine, act exclusively on the motor end-plate and do not affect the muscle fiber elsewhere. Here again the question must be asked, do these findings mean that the acetylcholine system or more specifically, the cell constituents upon which these quaternary compounds act, are absent in the conducting membrane of the muscle fiber or is there too a permeability barrier protecting these cell constituents against the quaternary nitrogen compounds applied externally? Prostigmine does not affect the response to direct stimulation of the muscle fiber, after complete curarization of the motor end-plate to exclude any effect through this junction. In contrast, eserine and DFP block this response (Couteaux *et al.*, 1946). We thus have the same difference between reactions of the muscle fiber to tertiary and quaternary nitrogen derivatives as described above with nerve fibers.

Finally, a new and conclusive confirmation is provided by the recent observations, discussed above, that when lipid-soluble analogs of acetylcholine are applied to the conducting membrane of nerve or muscle fibers in low concentrations, they act as postulated by theory.

If acetylcholine applied externally cannot penetrate into the fiber, because of a structural barrier, the ester released internally will not be able to leak through the barrier to the outside. This accounts for the observation that following stimulation of nerve fibers, acetylcholine appears in the perfusion fluid of synaptic junctions only. However, more that 20 years ago Calabro (1933) and Bergami and his associates reported that if a fiber is cut, acetylcholine is released during activity from the cut surface into the surrounding fluid (Bergami, 1936a, b; Bergami *et al.*, 1936). Their findings were repeatedly confirmed and extended to several types of nerves. Brecht and Corsten (1941) have demonstrated a release of acetylcholine even from cut sensory nerve fibers, although the quantity is smaller, as might be expected on the basis of various observations. These results indicate that acetylcholine is released during activity at points of the axon just as well as at the synapse, but does not penetrate into the outside as long as the structural barrier is intact.

2. *Curare*

The permeability barrier explains the limitation of the action of curare to the motor end-plate, as was first observed by Claude Bernard, a fact which has been so influential in promoting the idea of a special mechanism of transmission entirely different from that of conduction. Recent studies have revealed the structure of D-tubocurarine chloride (King, 1935; Wintersteiner and Dutcher, 1943). Two quaternary nitrogen groups situated at a distance of about 13 to 15 A. seem to be the active principle of curare and curare-like compounds. During the last decade, these compounds have been intensively studied by many investigators and have proved to be of great value in clinical use. A new development started with the synthetic preparation of compounds comparable in their action and structure to natural curare products by Bovet and his associates (Bovet *et al.*, 1946). During the following years Bovet and Bovet-Nitti (1948; Bovet *et al.*, 1949a) and their associates prepared a great number of curare-like compounds ("Curari di Sintesi") and thoroughly investigated in their classical work the relationships between structure and pharmacodynamic actions. Bovet (1951)

and his colleagues (Bovet *et al.*, 1951) introduced a distinction between two types of curare-like compounds with diametrically opposed effects. They define Leptocurares (linear molecules and relatively small) which may have a short depolarizing and stimulating action similar to acetylcholine itself, followed by a block, and Pachycurares (relatively voluminous molecules, like the curare itself) which antagonize the action of acetylcholine and exclusively block synaptic transmission without depolarization. Contrary to the view of other investigators, Bovet emphatically maintained that both types must act by the same fundamental mechanism, in spite of the apparent opposite effects, i.e. by competition with acetylcholine. This view has found strong support by further developments and will be discussed later in more detail. Of particular interest in the group of Leptocurares turned out to be succinylcholine. Bovet and Bovet-Nitti and their colleagues (1949b) were the first to describe the curarizing action of this compound and to recognize the close structural similarity to acetylcholine. The molecule resembles two acetylcholine molecules linked together. The compound proved to be invaluable in surgery, being today widely used in anesthesia (Bovet and Bovet-Nitti, 1955).

The evidence for the assumption that the block by curare must be attributed to the action on the receptor and not to that on acetylcholinesterase will be discussed below. Diquaternary ammonium salts are much more strongly bound to acetylcholinesterase than are the corresponding monoquaternary salts. The binding increases by a factor of more than 100. A combination of increased Coulombic and unspecific van der Waals' forces may readily account for this increase in binding, if one assumes two negative charges in the protein surface at an appropriate distance. The strong competitive action between curare and acetylcholine for the receptor is consistent with the idea that the various proteins reacting with acetylcholine, in this case the receptor protein, have similar active groups in their surface.

If monoquaternary ammonium salts are unable to penetrate the structural barriers surrounding nerve and muscle fibers, it is certainly to be expected that diquaternary salts will not enter. This does, of course, not mean that the acetylcholine receptor upon which curare acts at the myoneural junction is absent in the axon; it only cannot be reached. A structure containing two cationic nitrogens at the proper distance, but capable of penetrating the barrier, should, however, have a curare-like action. This is indeed the case

as has been shown in experiments with stilbamidine. This compound is a diamidine derivative and has two cationic nitrogens at a distance of 12 to 15 A. Stilbamidine inhibits acetylcholinesterase in concentrations of the same order of magnitude as analogous curare-like diquaternary ammonium salts. It blocks in low concentrations transmission across the neuromuscular junction. In contrast to curare, a small fraction of the compound at neutral pH is uncharged. One could, therefore, expect that stilbamidine in contrast to other diquaternary ammonium salts would be able to penetrate into the interior of the axon and act on the active membrane. In view of the smallness of the uncharged fraction, higher concentrations of the compound would be expected to be necessary to block axonal conduction than to block the neuromuscular junction. Experiments with stilbamidine on conduction have borne out this expectation. Block of conduction has been readily obtained on axons (Bergmann *et al.*, 1950a, b). A block of the direct spike has also been obtained with the curarized single isolated electroplax preparation of *Electrophorus electricus* (Schoffeniels and Nachmansohn, 1957).

It appeared possible that quaternary compounds, once they are inside the axon, would affect conduction. To test this possibility, micro-injection techniques worked out by Kao and Chambers were used to inject various quaternary ammonium salts into the giant axon of squid. Several compounds tested in a series of preliminary experiments seemed to block conduction in extremely low concentrations (Grundfest *et al.*, 1952). More recently, however, Hodgkin and Keynes (1956) repeated the micro-injections with a more refined method. They did not observe any effect of curare in relatively low concentrations, about 10^{-5} *M*. It is possible that the technique of micro-injections used in the earlier experiments was inadequate and that the block observed was an artifact. The active membrane may be surrounded from both sides by a lipid layer. The question may be checked experimentally by comparing tertiary and quaternary inhibitors of acetylcholinesterase using the improved technique. These experiments would reveal whether tertiary compounds injected into the interior affect conduction and whether quaternary analogs are less active or not at all active even from the inside.

Injection of compounds into a cell is a rather crude technique to mimic or affect specific intracellular processes. An extraordinarily complex structural organization of the cell always has been suspected and

is now well supported by electron microscope studies. The existence of a great many barriers and the evidence that the cellular processes take place in special, organized sequences make it doubtful whether and how efficiently we can, by injecting a compound, affect a special structural and molecular arrangement in a way similar to that produced physiologically by the same compound released internally. Injection of ATP into the muscle fiber has, as it is well known, no effect. This does not exclude its rôle in muscular contraction. It is evident that negative experiments cannot be used to exclude the functional importance of a particular compound. The preparation of greatly simplified systems, referred to as models by H. H. Weber, such as his myosin threads and the glycerinated fibers of Szent-Györgyi, made possible the demonstration of the specific ability of ATP to produce contraction and opened the way to study of the interaction between ATP and actomyosin. Similar reservations apply to the action of ATP injected into the squid axon for obtaining more information about the sodium pump. The free energy of this nucleotide, which is used in so many energy-requiring cellular processes, most likely plays an important rôle in the active ion transport, in the extrusion of Na^+ and uptake of K^+ *against* the concentration gradients. The amount of phosphocreatine which breaks down in the recovery period after the discharge of electric tissue (Nachmansohn *et al.*, 1943a, b), suggests that a considerable fraction, probably the greater part of this energy, is used for the restoration of the original uneven ion distribution. Only a small fraction of the energy is used for the resynthesis of acetylcholine. This view has found support in the recent observations of Whittam (1958) on the role of ATP in the active ion transport in red blood cells. In the same direction, although less directly, point the observations of Caldwell (1956, 1957) on the effects of dinitrophenol and cyanide on Na^+ efflux and on the phosphate esters. Caldwell and Keynes (1957) found an increased efflux of Na^+ after injection of ATP into the giant axon of squid poisoned with HCN. The effect is interesting but its significance is still uncertain, especially since both HCN and anoxia were found to increase Na^+ efflux in K^+-free solution. The experience gained in several decades of muscle research should be a warning against the formulation of too definite conclusions when one deals with such complex systems as an intact cell. The failure of del Castillo and Katz (1955a) to obtain an action of acetylcholine on the neuromuscular junction by intramuscular injections falls in the same category. The special structural organization

of cells makes it possible to reproduce and mimic a physiological event only under favorable conditions.

3. *The Origin of Acetylcholine Found in Perfusion Fluids*

The hypothesis of neurohumoral transmission assumed that acetylcholine is released from the nerve terminal and, crossing the nonconducting gap between nerve ending and muscle, acts as a mediator of the impulse on the effector cell. The significance of the appearance of acetylcholine in perfusion fluids in presence of eserine will be discussed later. But the original hypothesis would find at least some support if it could be demonstrated that the acetylcholine is released exclusively from the nerve terminal. It is, of course, exceedingly difficult to establish directly the site of origin of release, in view of the inaccessibility of the synaptic junction for direct measurements. In 1936, Dale and his associates presented for the first time observations which seemed to suggest the nerve terminal as the site of origin of acetylcholine found in the perfusion fluid. Motor nerves of cats and dogs muscle were cut and the nerve endings permitted to degenerate; after direct stimulation of the muscle, the authors failed to detect acetylcholine in the perfusate (Dale *et al.*, 1936). This failure has been interpreted as evidence that the acetylcholine found in the normal perfusate must originate from the nerve terminal, although this evidence is in the best case only a very indirect one and permits other interpretations.

These experiments were repeated by McIntyre and his associates. (McIntyre *et al.*, 1950). They found in contradiction to the earlier findings that even after complete degeneration of the nerve terminals, acetylcholine appears in the perfusion fluid of denervated muscle when fibrillations take place. At a recent symposium on curare and curare-like substances at the Institute of Biophysics at Rio de Janeiro, McIntyre (1958, 1959) again described studies on the acetylcholine content in the eserinized perfusion fluid of denervated dog leg muscles and in that of the opposite, normally innervated leg muscles, before, during, and after application of stimuli to the sciatic nerve. In a number of experiments, the postganglionic autonomic nerves were extirpated at the time the sciatic nerve was cut. The perfusates from indirectly stimulated muscle and the perfusates from the opposite denervated muscle of the same animal were found to contain amounts of acetylcholine of the same general order of magnitude, whereas the perfusates obtained from innervated muscle at rest contained only minute amounts of acetyl-

choline or none. In discussing the contradiction to the earlier findings and a possible explanation for the previous negative results, the author points to several factors which may result in failure to detect acetylcholine in perfusates. The sample of perfusate collected was possibly not derived from that perfusate which has actually traversed the muscle capillaries. With the use of India ink in the perfusate and histological examination at the end of the experiment, considerable variations were found as to the distribution in the capillaries. When the ink was uniformly distributed in normal or denervated muscle, the perfusates did contain acetylcholine; when, however, the ink was poorly distributed, little or no acetylcholine was found. The denervated preparations appear to be more variable as to the state of capillaries than the normal controls on the opposite site of the same animal. Since the presence of eserine results in a release of adrenaline, noradrenaline, and serotonin from the adrenal medulla, and the denervated muscles are hypersensitive to these compounds, McIntyre suggests the possibility that these factors may account for the previous failure to detect acetylcholine in denervated muscles. When all precautions were taken to ensure obtaining accurate quantitative estimates in the light of these sources of error, acetylcholine was found to be present in the perfusate of the denervated muscles. These data and a number of physiological tests, including studies of the effects of D-tubocurarine, led McIntyre to the conclusion that the motor nerve is not necessary for the elaboration of acetylcholine. Thus, the only experimental evidence ever offered in support of the assumption that acetylcholine is released exclusively from the nerve terminal has been questioned and explained in a way contrary *to the postulate of the hypothesis of neurohumoral transmission.*

In summary, the early evidence in favor of neurohumoral transmission presented in the thirties supports the assumption that acetylcholine must have an important rôle at synaptic and neuromuscular junctions. This assumption was, and still is, of great value for physiology and pharmacology. It has drawn the attention of many investigators to this compound and stimulated many studies of this phenomenon. Much new and valuable information has been obtained about many pharmacological aspects of the junctions. But the data failed to satisfactorily answer the question of whether acetylcholine acts as a neurohumoral transmitter or whether electric currents are the propagating agents across synapses as well as in axons. The interpretation of the observations thus required new methods of approach.

A period of 20 years has elapsed and the huge amount of chemical and biochemical data and their relationship to function accumulated since then in favor of an essential role of acetylcholine in generating currents in the axon has been presented. We have next to consider various other types of information about synaptic and neuromuscular junctions which have been obtained during the last two decades. Most of the huge amount of new knowledge is outside the scope of this article. Only those findings which appear pertinent for the interpretation of the mode of action of acetylcholine in synaptic and neuromuscular junctions will be briefly discussed, especially the problem of how justified is the assumption of two different types of function of acetylcholine in the two phases of propagation.

B. Concentration and Localization of Cholinesterase at Junctions

1. *Chemical Determinations*

Let us first consider the information available as to the concentration and distribution of cholinesterase at synaptic and neuromuscular junctions. Whatever the exact mode of action of acetylcholine may be at these foci, it was necessary to postulate that the compound must be rapidly inactivated by an enzyme. This was first recognized by Dale in 1914, and in 1936, he and his associates stated that one of the chief difficulties encountered by the theory of neurohumoral transmission across synapses and neuromuscular junctions was the necessity of a removal of the acetylcholine liberated with a "flash-like" suddeness.

It was this problem, i.e. the question whether or not the rate of inactivation of acetylcholine by cholinesterase at junctions is adequate to permit the assumption of an active rôle of acetylcholine in the transmitter process, which stimulated the interest of the writer and led him in 1936 to initiate his studies on cholinesterase with the technical help of his assistant, Miss Annette Marnay. The concentration of cholinesterase was determined in the pelvic end of frog sartorius, which is free of nerve endings, and compared to that in a middle part rich in nerve endings, a procedure introduced by Keith Lucas (1907) in his physiological studies. The part rich in nerve endings has an esterase concentration 3 to 4 times as high as that in the pelvic end in which endings are absent. The relationship between the concentration at the junction and that in the muscle tissue proper was at that time incorrectly estimated on the assumption that the enzyme is evenly distributed in the

nerve fiber and in muscle tissue proper; it was only a few years later that the remarkable localization of the enzyme near the surface was discovered. This finding made necessary a re-evaluation of the relationship between enzyme concentration in muscle and nerve fibers and that in the end-plates. (For a more detailed discussion see Nachmansohn, 1959). But since the number of motor end-plates in frog sartorius was known from the studies of Pézard and May (1937), the determinations made permitted a rather precise evaluation of the absolute activity of the enzyme at the level of the neuromuscular junctions of these muscles. At a single motor nerve ending, 2×10^{-9} µg. (8×10^{9} molecules) of acetylcholine may be hydrolyzed during the refractory period (5 msec.) (Marnay and Nachmansohn, 1938; Nachmansohn, 1939a, b). The presence of a high enzyme concentration at synaptic and neuromuscular junctions was subsequently demonstrated by the writer in many ways and extended to a great number and great variety of types of tissues (Nachmansohn, 1939b, 1955). However it soon became apparent as mentioned before, that the concentration of the enzyme is high in all conductive fibers and that it is only *relatively* higher at junctions, usually by a factor of 3 to 5 times.

When the picture of an intramembranous action of acetylcholine emerged from the biochemical studies, a more precise localization of the enzyme at the junctions became of special interest for interpreting the mode of action of the ester at these junction. Is the enzyme highly localized at the nerve terminals, or in the postsynaptic membrane, or in both? If the mode of action in junctions is basically similar to that in axons, the enzyme should be present both in nerve terminals and in postsynaptic membranes, although the ratio of the concentrations at the two sites may vary according to structural and functional variations from synapse to synapse, from species to species, etc. If on the other hand, acetylcholine does not function at the presynaptic membrane but is only released there, and acts on the opposite, i.e. the postsynaptic side, the presence of the enzyme in the terminal would raise difficult questions as to its function and significance. Why should there be a powerful mechanism of removal if the compound acts on the other side? Would the esterase in the terminal not interfere with the efficiency of the process by acting on the ester before it reaches its point of destination? On the other hand, the presence on both sides, while not proving the point, would support a similar function of the system at the two locations.

The investigations of the last two decades have supplied the evidence that the enzyme is present on both sides of the junction. In 1939, Couteaux and Nachmansohn (1940; Couteaux, 1942) found that following the section of preganglionic fibers, a rapid decrease of activity takes place in the superior cervical ganglion of cat during the first week; it coincides with the disappearance of the nerve terminals. The amount of acetylcholine split per gram tissue (wet weight) per hour is about 500 to 600 mg. in normal ganglia and falls to about 250 mg. in ganglia in which the terminals had disappeared. In the preganglionic fibers, the enzyme activity amounts to about 60–70 mg. of acetylcholine split per gram per hour. The decrease per unit weight coinciding with the disappearance of the nerve terminals is much higher than one would expect on the basis of the enzyme concentration in the fibers. This discrepancy suggests that the concentration of acetylcholine in the nerve terminals inside the ganglion is several times as high as in the preganglionic fibers. One explanation appeared to be that the enzyme is localized in or near the surface membrane. In that case, the increase would be attributable to the extensive endarborization of the presynaptic fiber within the ganglion and the consequent great increase in surface area per unit volume. These observations led the writer to test, in the following year, in 1940, the localization of the enzyme in the giant axon of squid. The clear-cut result demonstrated that the enzyme is localized exclusively in the sheath and is completely absent in the axoplasm (see preceding section).

The high concentration of esterase in nerve terminal is also indicated by the experiments of Nachmansohn and Hoff (1944) on the spinal cord of cats. The enzyme concentration was determined after unilateral and bilateral deafferentation of dorsal and ventral roots. A marked decrease was found in the four segments of the spinal cord coinciding with the loss of nerve terminals. It is of special interest that the decrease in the dorsal segments was higher than might have been expected from the concentrations of the enzyme in the dorsal roots; thus, the observations indicate that, as in the ganglion, the nerve terminals of sensory roots have a high concentration of enzyme per unit weight, higher than that found in the axon of dorsal roots. This result must again be attributed to the endarborization.

In amphibian sartorius muscle, Feng and Ting (1938) reported a decrease amounting to about 30% of the muscle esterase within 4 weeks after section of motor nerves. This is the period of time required for

degeneration of nerve fibers in these muscles. During this time, no change in activity took place in that part of the muscle which was free of nerve endings. The changes of cholinesterase concentration at motor end-plates of guinea-pig gastrocnemius after section of the motor nerve were studied by Couteaux and Nachmansohn (1940; Couteaux, 1942). The estimates in this case are complicated by the considerable loss of weight of these muscles, so that the total concentration of the enzyme appears to have increased. Correcting for the factor of volume change it was estimated that the amount of esterase which disappeared at the motor end-plate within the first week after section of the nerve, i.e. the period of time during which the nerve terminals disappear, is about 10% of the total enzyme concentration present at these junctions. Four weeks after section, the enzyme concentration had decreased by about 50%, but was still high and remained high for prolonged periods of observation. The estimates in denervated muscles of guinea-pig are complicated by several factors. There is a huge amount of "inactive" tissue in relation to the active sites; the inactive mass changes greatly with the course of time; the amount of nerve terminals at the motor end-plate of this particular muscle is rather small. The estimate of a decrease of 10% during the disappearance of nerve terminals is therefore less precise than the figures obtained for the terminals of ganglia and spinal cord and for those of other muscles, although it still indicates the presence of the enzyme at nerve terminals.

Stoerk and Morpeth (1944) followed the changes of acetylcholinesterase concentration in the gastrocnemius of rats after section of the sciatic nerve. They found that within 2 days, one-third of the enzyme disappeared; the remaining activity remained essentially the same over a period of 3 weeks. These biochemical data thus indicate (in good agreement with the other results reported) a high concentration of cholinesterase both in the nerve terminals and in the postsynaptic structure. The ratio of the two fractions varies considerably, as might be expected from the variations in morphology.

2. *Histochemical Data*

The introduction of new histochemical and staining techniques has brought valuable new information. The development is discussed by R. Couteaux in the preceding chapter, and only a few aspects may be mentioned very briefly here, insofar as the results have a bearing on the discussion of the mode of action of acetylcholine in synaptic trans-

mission. Using postvital staining with such stains as Janus green B and others, Couteaux (1947) described, in the superficial layer of the sarcoplasm of the motor end-plate, a complex lamellar structure to which he referred to as subneural apparatus. This structure is situated at the deep face of the nerve branches wherever they come into contact with the sarcoplasm. When Koelle and Friedenwald (1949) described their histochemical method for determining esterase activity, using acetylthiocholine as substrate for acetylcholinesterase, they found, in confirmation of the biochemical data, the existence of a high enzyme concentration at motor end-plates. This interesting technique was then applied by many investigators for more precise studies of localization of the enzyme at motor end-plates as well as at many other sites. Several modifications of the technique became necessary before the results could be satisfactorily evaluated. Detailed discussions may be found in the lucid presentation of Couteaux (1955; Couteaux and Taxi, 1952). The interpretation of the results obtained with the histochemical staining techniques offered some difficulties in regard to the problem of the presence of the enzyme in the nerve terminals. With muscular preparations of the mouse, staining of the nerve terminals appeared only after a treatment with which diffusion artifacts may appear (Couteaux and Taxi, 1952). The authors consider it possible, therefore, that the picture obtained is the result of a diffusion of the reaction products. But the question remained open as to the presence of the enzyme in the presynaptic membrane; in view of its vicinity to the subneural structure (see the electron microscope picture in the following section), the method did not permit a distinction. Coërs (1953), following with Koelle's method the changes of cholinesterase activity at the motor end-plates of rat gastrocnemius muscle after denervation, found, however, a weakening in the intensity of staining after degeneration of nerve terminals, indicating an important diminution of activity. Recently, Koelle and Koelle (1958) found definite evidence for the localization of acetylcholinesterase in axon terminals of various ganglia of cat, by applying a new procedure in which staining techniques were used in combination with reversible and irreversible enzyme inhibitors (Koelle, 1957) and by comparing ganglia before and after denervation. The histochemical data thus are in agreement with the biochemical data; both clearly indicate the presence of acetylcholinesterase at both pre- and postsynaptic structures.

C. Differences between Axon and Synapse

1. *Structure*

Let us turn to various other aspects of the synaptic junctions pertinent to the problem under discussion. As stated so correctly by Couteaux (1955), the morphology and physiology of the neuromuscular junction have long been studied almost independently of each other. In recent years, serious attempts have been made to bridge the gap. For a satisfactory interpretation of the mechanism of synaptic transmission, knowledge of structure and ultrastructure are indispensable. During the last few years, extensive electron microscope studies have greatly advanced our knowledge of cell membranes, including those of nerve and muscle, and have contributed to a better picture of the ultrastructure of myoneural and synaptic junctions (Robertson, 1956, 1957a, b, c; Reger, 1957; Palade and Palay, 1954; Robertis and Bennett, 1955; Luft, 1956; Edwards *et al.*, 1958). The latest studies of Robertson (1957a, b) indicate that cellular membranes, including those covering the nerve fibers, terminals, and the muscle fiber, are about 75 A. wide. Three layers may be distinguished in these membranes. There are two parallel dense layers of about 20 A. width, separated by a less dense layer. There is some reason to believe that this latter layer may contain a bimolecular leaflet of lipid, whereas the more dense layers may contain proteins.

The electron microscope picture of the myoneural junction is illustrated by Fig. 7, which is an interpretive diagram of the myoneural junction of lizard, by Robertson (1956). The small branches of axon terminals lie in troughs in the surface of the muscle fibers. The muscle surface membrane in these troughs is formed by complex branching and anastomosing folds; they are identical with the subneural apparatus of Couteaux. A compound membrane 500 to 700 A. thick separates axoplasm from sarcoplasm. This compound membrane referred to by Robertson as synaptic membrane complex consists of five distinct layers. The middle layer was for a time interpreted by Robertson to be the continuation of the outer membrane covering axonal and muscular fibers. Recently, he is inclined to consider it as an artifact, resulting from a precipitation of the ground substance.

Several features are still interpretative and need further clarification. But looking at the picture, it appears quite obvious that such a complex morphological structure must greatly affect the electrical events. It

appears likely *a priori* that certain properties and consequently the electrical manifestations of such a structure should show marked deviations when compared to those in a simple cylinder such as the axon. It may be recalled (Eccles, 1946) how electric fields and the response to them are dependent on the "geometry" when studied in such simple structures as axons. No information exists about the effects of geometry on electrical events in structures of such a complex nature as are here discussed.

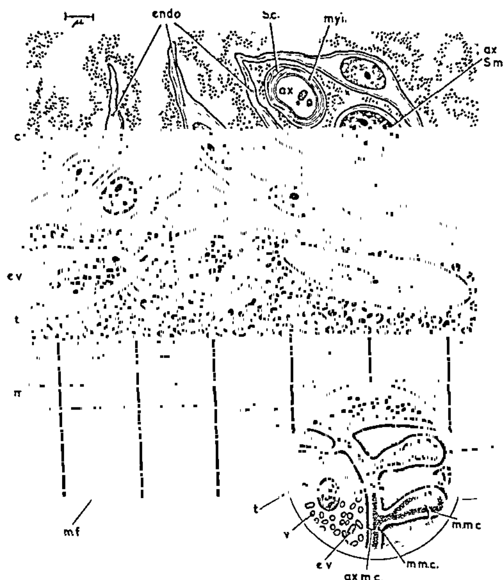


FIG. 7. An interpretative diagram of a myoneural junction in lizard, taken from Robertson (1956). The manner in which the muscle surface complex is thrown into the junctional folds of the neuronal apparatus is indicated. The region marked by the circle is enlarged to show more detail. The layers of the synaptic membrane complex are shown between the arrows, syn. m.c. (For details see original paper).

An interesting finding of electronmiscroscope studies are the vesicles, (Robertis and Bennett, 1955) present in great numbers in terminal portions of neurons. They have also been found in postsynaptic structures, in Schwann cells (Robertson, 1957c), and near Ranvier nodes. Recently, "aposynaptic" granules have been described on the postsynaptic side of the junction in insects (Edwards *et al.*, 1958). A considerable amount of literature exists already about the chemical aspects of these vesicles. It has been proposed that they contain packages of acetylcholine. The number of acetylcholine molecules per vesicle has been estimated. Physicochemical mechanisms as to how these packages of acetylcholine are released from the vesicles through the terminals during activity have been proposed. The formation and hydrolysis of acetylcholine by choline acetylase and cholinesterase has been envisaged to be related to variations of the vesicles observed with the electron microscope. Accustomed to more conservative and orthodox methods of chemistry and biochemistry, the writer finds it difficult to evaluate biochemical events and physicochemical mechanisms exclusively on the basis of electron microscope pictures.

2. *Electrical Signs. The End-Plate Potential*

Göpfert and Schaefer (1938) discovered the existence of a special end-plate potential. Its characteristics were elaborated by Kuffler (1942). Since then, a considerable number of studies have been done on the various features of this and other synaptic potentials and the effect of drugs upon these potentials. The technique of penetrating frog muscle fibers with microelectrodes, glass pipettes filled with KCl and having a tip less than 1 μ in external diameter, introduced by Ling and Gerard (1949) and further developed by Nastuk and Hodgkin (1950), greatly helped detailed investigation of the postsynaptic potentials. For these studies, the microelectrodes are inserted in the immediate vicinity of the end-plate, the other electrode being outside the muscle fiber. The action of acetylcholine on the potentials was studied with this technique by Fatt and Katz (1951) and has been since then the subject of particularly numerous and extensive investigations. It is today generally agreed that the end-plate potentials are produced by the action of acetylcholine and that the ester has there a depolarizing action, as was suggested a long time ago by Dubuisson and Monnier (1934) and Cowan (1936) and experimentally supported by the depolarizing (electrogenic) action demonstrated in 1939 on the *Torpedo*, as men-

tioned before. Further progress was achieved when Eccles and his associates (Brock *et al.*, 1952a, b) succeeded in inserting microelectrodes into motor neurons. A detailed discussion of the electrical characteristics of the postsynaptic potentials may be found in various reviews (e.g. Fatt, 1954; del Castillo and Katz, 1956) and in a recent book of Eccles (1957).

Nerve terminals are inaccessible for corresponding studies with inserted microelectrodes; but few electrophysiologists will be willing to assume that the negative waves sweeping down the axon stop before the nerve terminal, and that there is no presynaptic potential. This leaves us at present with the necessity to speculate about various aspects of this potential. What is the basis for the synaptic delay, which is of the order of magnitude of 0.5 msec. Is it due to the slowdown of propagation in the fine terminal filaments or to the events taking place directly at the junction, whatever their nature, or to both? The delay cannot be attributed to a diffusion of a chemical substance from the pre- to the postsynaptic membrane. The high temperature coefficient of the latency period (high energy of activation) excludes diffusion as a major factor responsible for the synaptic delay.

Do the more recent electrical data offer a possibility of a definite decision between the two opposing views, the one accepting neurohumoral transmission in its original form and the other assuming that currents are the propagating agent and that release of acetylcholine takes place in both pre- and postsynaptic membranes? The failure to demonstrate experimentally electrotonic spread from the terminal has been used as an important argument in favor of the assumption of neurohumoral transmission. But in their recent review, del Castillo and Katz (1956) admit the weakness of such reasoning. Attenuation of subthreshold electrical signal in the fine terminal filaments may be much more severe than in larger and more accessible parts of the system. Therefore, the signals might have faded out before reaching the junction. In fact, one may repeat here the pertinent question of Erlanger (1939) and include the new information about the actual distance involved. Since electric currents are known to be able to cross a nonconducting gap of more than 1 mm, is it reasonable to assume that they would not be able to cross the distance of 500 to 700 A. between pre- and postsynaptic membranes? Electrotonic spread is inadequate to propagate the impulse along the axon. Forty years ago, Lucas (1917) and Adrian stated that locally supplied energy is neces-

sary at successive points of the axon during propagation. If this is true for the axon, it must apply for the junction. If the ionic concentration *gradients are the local source of energy for currents propagating the* impulse along axons, and this potential source is made available at the excited points by the action of acetylcholine, the currents generated in the presynaptic terminal may be generated in a similar way and, *reaching the postsynaptic membrane, they may mobilize acetylcholine* to produce the postsynaptic potential. Action currents of fibers are able to stimulate adjacent fibers under appropriate conditions and suitable composition of the outer milieu. Such favorable conditions may *prevail at the junction.*

In this connection, the interesting observations of Bullock (1948) and Bullock and Hagiwara (1957) on the giant synapse of the stellate ganglion of squid may be mentioned. This synapse, because of the large size of the pre- and postsynaptic structures, permits recording of the presynaptic spike and of the postsynaptic response. There is a synaptic delay of 0.5 msec. at 22°C. and 2 msec. at 10°C. Only an insignificant amount of current (100–300 μv.) can be detected passing between the two structures. These experiments raise many questions of general interest; the interpretation of the possible mode of transmission is difficult, however, as long as the ultrastructure is not well established, which is the case at present. There is a possibility that the pre- and postsynaptic membranes are fused at some points at which there would then be no extracellular space. This would, of course, create very unusual and peculiar conditions, since propagation across these points could not be conceived in terms of any known mechanisms and might instead well proceed just around them.

Inhibitory synaptic activity has long been known. Various explanations were given, based on indirect evidence, such as the depression of a testing reflex discharge. Bernhard *et al.* (1947) demonstrated that conduction of impulses by certain spinal pathways may result in excitatory effects in some motor neurons, inhibitory effects in others. New information has been obtained with microtechniques that permitted intracellular recording. Studying with these techniques the inhibitory actions on motor neurons, Eccles and his associates observed a transient increase of the membrane potentials, i.e. hyperpolarization (Brock *et al.*, 1952a, b). The potentials have been designated as inhibitory postsynaptic potentials in contrast to the excitatory postsynaptic potentials. Synaptic inhibitory action on crustacean stretch receptor was shown

by Kuffler and Eyzaguirre (1955) to be attributable to a repolarizing action exerted on cells which had been depolarized by stretch. Several similar effects, or slight variations, of inhibitory mechanisms were described for other junctions with the use of microelectrodes. The inhibitory action of the vagus on the heart has long been known, since Gaskell (1886) demonstrated increased demarcation potentials of the cardiac muscle which accompanies stimulation of the vagus nerve. Hyperpolarization and decreased action potential were shown to be produced by acetylcholine or carbamylcholine applied to a strip of muscle from cat's auricle (Burgen and Terroux, 1953). These observations were confirmed by del Castillo and Katz (1955a) and by Hutter and Trautwein (1955). Inhibitory mechanisms at the neuromuscular junction of crustaceans were observed by Fatt and Katz (1953b). A large and prolonged postsynaptic hyperpolarization evoked by a presynaptic stimulus was observed by Laporte and Lorente de Nó (1950) in the deeply curarized cervical ganglion of turtle.

The phenomenon of inhibition by hyperpolarization greatly surprised and impressed Eccles and was apparently an important factor in his famous "conversion" to the neurohumoral transmitter theory. It is certainly good reasoning to attribute hyperpolarization to a chemical reaction. Hyperpolarization is unexplainable in purely physical terms. But so is depolarization. Both phenomena require chemical processes in the active membrane, regardless whether they occur in axons or at synapses. However, the fact that the phenomena are produced by chemical reactions does not give a clue to the problem how they are initiated. They may be mobilized by current flow from the pre- to the postsynaptic membrane. The amperage of the currents generated by the presynaptic fiber would, according to Eccles (1957), fail by a factor of 50 to produce the current required for activating the postsynaptic membrane of motor neurons, even if the presynaptic fiber could very effectively inject its currents across the postsynaptic membrane. In motor end-plates of amphibian muscle, the factor may be as high as 1000. This, of course, requires mobilizing chemical energy. Again, it does not reveal the mode of activation and the chain of events by which a small effect initiates a chemical reaction. This action may eventually have very powerful consequences. The energy of the electric wave propagated along the muscle fibers may e.g. release amounts of energy of contraction many thousand to a million times greater. Many other examples could be given.

Variations of the postsynaptic membrane potential by extrinsic current causes large changes and even a reversal of the inhibitory postsynaptic potentials. This observation may rather be used as an argument against neurohumoral transmission than for it, because it indicates the possibility that intracellular or intramembraneous chemical changes produced by extrinsic current may be responsible for variations of hyperpolarization. Currents flowing from the pre- to the postsynaptic membrane may then just as well account for initiating the chain of events leading to those changes. Special structural arrangements may make the small eddy currents at junctions extremely efficient in initiating the chemical reactions responsible for the changes in the postsynaptic membrane which lead to either hyper- or depolarization. The point will be further discussed in connection with the permeability changes occurring during ion movements.

Of interest is Eccles' discussion of the observations on the electric response of deeply curarized ganglia, first reported by Laporte and Lorente de Nó (1950) and later studied by other investigators. Two phases were found, first a hyperpolarization, then a depolarization. Eccles admits that this finding presents a difficult and unresolved problem that defies simple explanations in the usual terms of neurohumoral transmission. He attributes the hyperpolarization, as usual, to some unknown transmitter. But why is the depolarization effectively depressed by all anticholinesterases, and why not by D-tubocurarine and its analogs? Eccles suggests a new type of cholinergic transmission, wherein acetylcholine depolarizes the ganglion cells by acting on special receptor areas having a cholinesterase configuration. It must be envisaged, *according to Eccles, that a single transmitter substance* may act on two completely different receptor surfaces on the same subsynaptic membrane. In this instance, Eccles becomes aware of the complexity of chemical systems. It is, of course, easy, to propose many alternative explanations. Whatever the interpretation may be, Eccles recognizes that the action of acetylcholine should not have under all conditions the same effect and proposes that in addition to the active ester, other members of the system must be considered. Since, therefore, such reactions are unpredictable so long as so little is known about the other factors involved, interpretations of detailed mechanisms based on electric signs only should be postponed until more information becomes available as to chemical processes involved.

3. *The Site of Drug Action*

The powerful effect of acetylcholine and analogous compounds on synaptic junctions was one of the two essential facts which formed the basis of the neurohumoral transmitter theory. It was assumed without much questioning that acetylcholine is released from the nerve terminal and acts on a receptor in the second cell. The crucial question as to the precise site of action was not even raised. Are these agents really acting only on the postsynaptic membrane? If an alternative mode of action is considered, i.e. the possiblity of an intracellular process taking place in pre- and postsynaptic membranes, both sites might be affected and the receptor should be present in both membranes. The receptor may be present in smaller quantity, the sensitivity may differ for various reasons, but the critical question still remains whether or not it is present in both membranes.

Eighteen years ago Masland and Wigton (1940) reported the important observation that prostigmine applied to the motor end-plate produced rapid bursts of electrical activity in the motor roots in addition to its action upon the muscle fiber. Curare blocked both muscle and nerve response. The authors concluded that their observations provide evidence that acetylcholine and prostigmine stimulate the motor nerve terminal as well as the muscle; the concept of a specific local effect of acetylcholine on the muscle at the myoneural junction is, as emphasized by the authors, insufficient to explain the findings. Most investigators, and the writer is one of them, did not recognize the startling implications of their findings, the evidence for an acetylcholine receptor in the terminals. Masland and Wigton's observations were confirmed in 1942 by Eccles and associates (1942) .These authors stated that there was no doubt that nerve discharges observed in presence of eserine are independent of, and prior to, the muscle impulses. In the experiments of Laporte and Lorente de Nó, curarization was mainly caused by a modification of the presynaptic fibrils and resulted in a block of conduction in the presynaptic arborization; this again points to the presence on that site of a receptor for acetylcholine with which curare competes.

The problem of the site of action of compounds applied externally and acting on the myoneural junction has been recently taken up by Riker and his associates (Riker *et al.*, 1957). They tested the effects of 3-hydroxy-phenyl-trimethylammonium ion and of various analogs such as phenylethyl dimethylammonium ion (tensilon), on the motor end-

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effects of prostigmine in producing contractures of the frog rectus muscle. The assumption of a direct action upon the receptor is supported by observations of Riker and Wescoe (1946) who obtained acetylcholine-like contraction with prostigmine after the enzyme had been strongly inhibited by alkylphosphates. The difference of action of prostigmine and eserine on the end-plate potential described by Eccles and MacFarlane (1949) also suggests—as has been pointed out by Riker (1953)—an action of the quaternary compound which cannot be explained exclusively in terms of esterase inhibition. The studies on the electroplax have also shown a block of conduction by eserine and prostigmine before a critically low level of the enzyme had been reached (Altamirano *et al.*, 1955).

The extensive studies on the pharmacological action of 3-hydroxy trimethyl-phenylammonium ion and its analogs revived the discussion as to the point of action. Nastuk and Alexander (1954) have emphasized the role of inhibition of cholinesterase in which potentiation is observed in presence of tensilon. Tensilon is a very potent inhibitor. The enzyme-inhibitor dissociation constant was found to be 3×10^{-7} *M*, while that of prostigmine was 1×10^{-7} *M* (Wilson, 1955). Wilson also found that the compound reacts very quickly with the enzyme, within seconds. The rapidity of the onset of potentiating action is therefore consistent with the interpretation proposed. On the other hand, the rapid anticurare action of these compounds is more difficult to explain in terms of enzyme inhibition. The absence of a depolarizing action in concentrations which counteract curare permits several alternative interpretations. An action on the nerve terminals, which had been already considered as a possibility by Nastuk and Alexander, has now been demonstrated by Riker *et al.* A bimodal action, i.e. on enzyme and on receptor, appears likely. The pharmacological aspects are fully discussed in the papers quoted. In connection with the mode of action of acetylcholine at the neuromuscular junction, these developments were useful for directing the attention toward the complexity of the drug effects. First, there is the evidence of a direct action of acetylcholine on the nerve terminals, requiring the presence of an acetylcholine receptor as well as the esterase in the presynaptic membrane; the evidence for the presence of the latter was discussed before. Thus, there are two sites of action, both the pre- and the postsynaptic membranes. The strength of the response may depend on additional factors influencing the relative sensitivities of the two membranes. Secondly,

plates of cat muscle. Riker *et al.* observed that the compounds applied to the junction produce repetitive discharges which may be recorded on motor roots. They conclude that these compounds must definitely act on a receptor in the nerve terminals. They postulate that acetylcholine is released from a bound form in the motor nerve terminal and reacts with a receptor *to initiate synaptic transmission*, a view which is in full agreement with that proposed by the writer many years ago.

An intermembraneous action of acetylcholine implies, as pointed out before, the location of all members of the system at close vicinity, structurally organized within a few molecular layers. A compound penetrating from the outside and affecting one member may then easily affect the others, depending on the degree of affinity, which may differ considerably among the members, in spite of similarity—but not identity—of the reacting molecular forces. Determination of affinity to proteins is more difficult when evaluated on intact cells than when evaluated on enzymes in solution, since the response of the living cell depends on a complexity of factors. The powerful action of acetylcholine and other analogous quaternary ammonium ions has always been associated with their action on a receptor substance. This interpretation has also been widely accepted for diquaternary curare-like nitrogen derivatives; they may have not only a blocking but also a stimulating action similar to or even stronger than that of acetylcholine. Paton and Zaimis (1949), for instance, found a depolarizing action by a series of diquaternary ammonium ions where the two trimethylated nitrogen groups were separated by a varying number of methyl groups. The C_{12} compound was the most effective. Although these diquaternary compounds have higher affinities to acetylcholinesterase than their monoquaternary analogs, the K_I being between 10^{-4} and 10^{-5}, while that of the monoquaternary is closer to 10^{-3} or even 10^{-2}, the effects observed were attributed to the action upon the receptor (Paton and Zaimis, 1949; Zaimis, 1951).

In spite of the evidence for the reaction of quaternary nitrogen derivatives with the receptor, effects of some of the quaternary compounds were interpreted exclusively in terms of acetylcholinesterase inhibition, in view of their high affinity to the enzyme as measured by their inhibitory action *in vitro*. During the last decade, however, much evidence has accumulated from pharmacological studies suggesting an action of this type of compounds on both members of the system. Aeschliman and Stempel (1946) and Lehmann (1946) reported direct

tative relations have been established for the action of drugs on cells, there probably remain dozens of unknown variables, and there is usually a considerable range of possible alternative explanations." Two decades ago hardly any data were available on the biochemical and physicochemical aspects of the acetylcholine system. The interpretation of the mode of action of acetylcholine, given at that time on the basis of its pharmacological effect, was justifiable. To maintain rigidly the original interpretation, to oppose or reject categorically any modification, appears less justifiable in view of the huge amount of knowledge acquired during the last two decades.

4. *Ion Movements and Permability Changes at Junctions*

Fatt and Katz (1952b) suggested that the efflux of acetylcholine from nerve terminals may be analogous to that of K^+ from the axon and that acetylcholine may leave the terminal by a specific exchange with Na^+ which enters the terminal during activity. Later they realized that this hypothesis was untenable and withdrew their suggestion. They now assume that the acetylcholine released from the nerve terminal acts on the postsynaptic membrane by increasing permeability to ions. The idea as to the function of the ester is essentially similar to that proposed twenty years ago by the writer for its rôle in conducting membranes, with the modification that the action is *intra*, and not *inter*-cellular. But in contrast to the events in the axonal membrane, in which a specific transitory increase in sodium permeability takes place, followed by a smaller and less rapid change in potassium permeability, the change in permeability of the postsynaptic membrane is assumed to be unspecific. This hypothesis is based on the following finding. When a muscle is immersed in isotonic K_2SO_4 solution, the membrane is completely depolarized; the preparation is inexcitable electrically and application of acetylcholine does not produce a potential change in either direction. When, however, the membrane potential is displaced from zero by the passage of current, renewed application of acetylcholine reduced the potential difference built up across the end-plate membrane (del Castillo and Katz, 1955b). This displacement of the potential difference is interpreted as an increased flux of K^+ and therefore it is concluded that acetylcholine increases in the postsynaptic membrane both Na^+ and K^+ conductances and possibly the permeability to other ions as well. Consequently, it is argued, acetylcholine cannot be responsible for the more specific permeability changes in the axon.

most compounds may affect preferably either receptor or esterase; or they may act on both. Action on the receptor may have again various consequences, dependent on structural properties of the compound. As we have seen, they may determine whether it will react with the receptor as an activator or as an inhibitor, i.e. produce depolarization and stimulation, or depolarization and block, or just block without depolarization. Finally, a quaternary compound may act with varying strength on the two other protein members of the system in addition to receptor and enzyme. Little consideration has been given to this possibility. All this new information emphasizes the necessity of revising the interpretation of many of the former observations.

The difficulty of interpreting cellular mechanisms on the basis of effects of drugs injected into blood vessels or applied to complex preparations is also suggested by the interesting observations of Kewitz and Reinert (1954a, b) on the discrepancy between factors affecting the action of acetylcholine on synaptic functions and those affecting physiological transmission. In perfusion experiments of the superior cervical ganglion of cat, they observed that the absence of glucose and lack of potassium decreased the response to injected acetylcholine; the response to electrical stimulation was not affected. In case of calcium deficiency, the two processes were changed in an opposite way. Acetylcholine action was greatly increased, while the response to electrical stimulation was abolished. It is impossible to discuss the many discrepancies reported or their possible causes. But these observations may suffice as illustration of the contradictions encountered. They should caution us against using too readily the effects of drugs, metabolites, antimetabolites, and ions as a means of explaining their rôle in a cellular mechanism. Actions of compounds on living cells frequently raise interesting problems; such tests may provide important information; but they permit conclusions as to the mode of action only to a limited extent and only in connection with other data. This has been often stressed by leading pharmacologists. In 1933, A. J. Clark wrote, "The physical chemist can reasonably hope to simplify his conditions and to reduce the number of variables, until he obtains a system that provides formal proof of the laws which he enunciates, but the pharmacologist is interested in the action of drugs on the living cell and any attempt to simplify this material results in death. Hence he cannot hope to obtain formal proof for his theories and must be content with intelligent guesses. Even in the most favorable cases where quanti-

(1957) injected tetraethylammonium ions into squid axons. The action potentials became greatly prolonged, but were abolished by the removal of Na^+. The effect of lipid-soluble quaternary ammonium ions has been discussed. How far some of these ammonium ions are able to affect the conducting membrane directly, and by which mechanism, has not been ascertained as yet. At present, these findings remain unexplained but raise interesting questions. They indicate the complexity of the problem and show how far we are from a real understanding of the mechanism of bioelectric potentials since we are at present unable to account for these observations. Interpretations in simple terms of Na^+ and K^+ movements have become untenable. Without a better knowledge of the chemical forces involved, the situation will hardly change.

Removal of Na^+ from the extracellular fluid blocks synaptic transmission as well as the effect of applied acetylcholine (Nastuk, 1954). This observation would point to similarities in the membrane properties and in the function of the ester at both synapse and axon. Nevertheless, it is entirely possible that, as a result of other factors, there are certain modifications in the effect of acetylcholine action on ion permeability in axonal and in synaptic membranes. But experiments of a completely different type would be necessary to substantiate such an assumption.

Accepting Fat and Katz's evidence that acetylcholine increases the permeability of the postsynaptic membrane to all ions and thereby produces the excitatory postsynaptic potential, Eccles (1957) proposes the hypothesis that the inhibitory postsynaptic potential in motor neurons is associated with an increased permeability for small ions only, ions of about the size of K^+ and Cl^-, Na^+ being excluded. He envisages the excitatory and inhibitory postsynaptic membrane as being momentarily converted into a sieve-like structure with two different sizes of pores. He does not suggest that the actual pores have these sizes, only that the effective size of the pores, as far as the passage of ions is concerned, differs. This interesting picture is at present purely hypothetical, based essentially on variations of potentials due to the intracellular increase of ions introduced from the micropipette under the influence of current pulses. In view of the hypothetical nature of these suggestions, the picture need not be discussed here until further explorations have been made. However, Eccles proposes that some hitherto unknown transmitter substance different from acetylcholine produces the opening of the small pores. Since acetylcholine is able to

High K^+ concentration drastically affects the membrane properties. Passage of transverse current must also influence them. Effects of acetylcholine applied under such extreme and unphysiological conditions may be entirely different from those of acetylcholine released within the membrane under physiological conditions. The ion movements across the axonal membranes during activity have been established with very elaborate techniques with the aid of radioactive material over a period of many years. Only with extremely delicate methods was it possible to demonstrate the differences of rates between Na^+ and K^+ fluxes during the rising and falling phase of the spike. With less sensitive procedures, only the balance would have been found, i.e. an increased Na^+ influx and K^+ efflux of the same order of magnitude since electrical neutrality must be maintained inside the fiber. This would then have suggested an equally increased permeability to both ions.

On the other hand, even if the action of acetylcholine is essential to the changes in membrane permeability, the ester cannot be the only factor determining the nature of the changes as discussed before. A *physiological response depends on a great number of factors:* external and internal environment; membrane properties; chemical reactions, some of which are associated directly with specific function, others more concerned with maintaining the system. Electric currents may strongly affect all these factors and influence the response in a variety of ways. Pharmacologists have long known that many compounds may stimulate or block according to a variety of conditions, such as ion concentrations, state of irritability and fatigue, and presence of metabolites, of hormones, and of vitamins, etc.

Accessory factors may differ in axon and synapse and may vary to some extent in different biological materials. An illustration is provided by the variations observed with quaternary ammonium ions acting on different types of fibers in the absence of Na^+. Lorente de Nó (1949) found that tetraethylammonium ions restore excitability of B and C fibers of frog nerves, which had been made inexcitable by Na^+ deficient solutions. The excitability of A fibers is restored by guanidinium and certain other quaternary ammonium ions (Larramandi *et al.*, 1956; Lorente de Nó *et al.*, 1957). Fatt and Katz (1953a) found that crustacean muscle fibers, after treatment with tetrabutyl ammonium ions, gave a prolonged heart-like action potential in the absence of all monovalent cations in the external medium. Tagasaki and Hagiwara

synaptic terminal has been discussed. A complete barrier surrounding the presynaptic terminal would present a serious difficulty for the transmitter theory. A receptor, however, might have been absent and the demonstration of its presence in the terminal is, therefore, significant. There may be quantitative differences depending on species, types of synapses, etc., in respect to both pre- and postsynaptic membranes and to differences between them, as is indicated indeed by structural and pharmacological observations.

If a compound applied externally readily affects a protein in a membrane, the same compound, if it is there a naturally occurring metabolite, should be able just as readily to pass into the surrounding fluid. If it is enzymatically metabolized at extremely high speed, as is the case with acetylcholine, we would expect that only a negligible fraction might escape into the outside fluid. But if the cellular enzyme action responsible for the rapid removal is inactivated, then of course a relatively large fraction may appear in the outside and may be collected in the perfusate.

According to the view that acetylcholine is essential for generating currents both in the nerve terminal and in the postsynaptic membrane, it would be expected that under optimal physiological conditions, either negligible amounts of acetylcholine or none at all would leak to the outside from both membranes. This leakage should greatly increase on addition of cholinesterase inhibitors. This is exactly what happens. Appearance of the ester under these conditions can, therefore, not be used either as evidence for neurohumoral transmission or against its intramembraneous functions.

A few observations demonstrating the appearance of the ester in perfusates may be briefly mentioned, since they not only played an important rôle in the earlier history, but are still at present frequently discussed as to their significance. It may be recalled that Otto Loewi encountered difficulties in reproducing the appearance of the vagusstoff with regularity and did not succeed in finding it when he used *Rana pipiens*. Asher (1925) and other investigators reported that they could confirm Loewi's finding only when the heart became "hypodynamic."

These apparent contradictions may well indicate that the diffusion of a detectable amount of acetylcholine is a sign of a slight damage of the membrane. Such damage is readily conceivable, on account of several factors. The membrane is unprotected and may be particularly vulnerable to all kinds of irritations. Dale and his associates were

produce both hyperpolarization and depolarization, it appears unnecessary to postulate another unknown substance to take the place of acetylcholine. Additional chemical forces and structural arrangements discussed previously may determine the character of permeability change. The reported effects of strychnine (Bradley *et al.*, 1953) support rather than contradict the assumption that acetylcholine action is associated with permeability changes in inhibitory effects. Strychnine has a high affinity to acetylcholinesterase; the dissociation constant of the enzyme inhibitor complex is about 2 to 3 $\times$ 10^{-5} (unpublished observations of the writer). The alkaloid may have a still higher affinity for the receptor. It blocks conduction in the giant axon of squid (Bullock *et al.*, 1946). Recently, it was found also to block in low concentrations the response of the single isolated electroplax to both neuronal and direct stimulation (unpublished experiments of Schoffeniels).

In view of our ignorance as to the molecular forces effecting the permeability changes, all these hypotheses are obviously of a purely speculative nature. The writer finds it difficult, therefore, to understand how speculations proposed as possbibilities on one page are used, frequently only a few pages later, as conclusive evidence either against the rôle of acetylcholine in conduction or for neurohumoral transmission at synaptic junctions.

5. *Significance of Acetylcholine Appearance in Junctional Perfusates*

The demonstration of the appearance of acetylcholine in junctional perfusates following activity was considered, in the thirties, as important evidence for its transmitter rôle in the original sense. This interpretation appeared further supported by the observation suggesting the origin exclusively in presynaptic terminals. This latter observation has, however, been questioned, as discussed before.

When those studies were made, no alternative explanation of the rôle of acetylcholine was envisaged. In the light of all the data accumulated in the last two decades in favor of an intracellular rôle of acetylcholine, the significance of the appearance of acetylcholine in junctional perfusates must be re-evaluated. In contrast to the axon, which is surrounded by special structural permeability barriers, both pre- and postsynaptic membranes of the junction appear to be pervious to acetylcholine and other quaternary ammonium ion derivatives. This is indicated by their response to quaternary compounds applied externally. The increasing evidence for such actions on the receptor at the pre-

ated axons of invertebrates, the excess of enzyme was found to be about fivefold; in frog sciatic nerve, it appeared to be higher, but the results were less accurate, since the measurements were made at the limit of accuracy of the method used. On the other hand, the excess of enzyme may be higher at synaptic junctions than in axons. Moreover, the period of time required for the removal of the ester released per impulse is not known; this period may be either greater or—what is more likely—less than 1 msec. This introduces another factor of uncertainty. But the order or magnitude is comparable to the minimal amounts found to produce a response. On the basis of autoradiography of end-plates of a cat diaphragm treated with radioactive curare or decamethonium, Waser and Lüthi (1956) calculated that 8×10^6 molecules were fixed per motor end-plate. This is a slightly smaller figure, but still not very far from the values obtained with quite different methods and quite different types of muscle.

On the other hand, the quantities of acetylcholine appearing in perfusates in presence of eserinized Ringer have been re-evaluated in very carefully controlled observations by Emmelin and MacIntosh (1956). The discrepancy between the two quantities, the amounts found in perfusates and the amounts required to evoke a response, has markedly decreased. A really quantitative evaluation of their ratio, however, is still extremely difficult. Let us consider the difference to be five- to tenfold, which is on the low side of the estimates of those investigators who support neurohumoral transmission. This may mean that, in presence of eserine, 10 to 20% of the acetylcholine required for activity and which is metabolized would escape from the membranes. This is still a small fraction. In a muscle contracting a few times, probably no extra lactic acid is formed. The ATP hydrolyzed may be restored from phosphocreatine and from the pool provided by the citric acid cycle. With increasing amounts of work performed, lactic acid is formed and begins to accumulate, and with more strenuous work leaves the cell and appears in the outside fluid. A great number of stimuli are required for detecting acetylcholine; the preparation is exposed by necessity to various unphysiological conditions. It must, therefore, even be expected, that part of the acetylcholine released escapes from the membranes located at the two sides of the myoneural junction especially when protected from attack by the esterase. If the compound released combines with the receptor within the same membrane the action will be much more efficient; it will require smaller quantities and will be faster.

puzzled by Kibjakow's (1933) early report of acetylcholine appearance in the perfusion fluid of ganglia, without addition of eserine. Kibjakow's perfusion methods may have been less elegant and more damaging for the membrane than those of Dale and his associates. Similarly, Lorente de Nó (1938), studying the appearance of acetylcholine in the perfusate of the superior cervical ganglion of the sympathetic and of the ganglion nodosum of the vagus, found the ester on stimulation of the ganglion nodosum and on antidromic stimulation of the sympathetic ganglia. He attributed this appearance to cellular damage and described histological observations supporting his views. Lorente de Nó rejected the perfusion method in principle as being inadequate to decide the function of acetylcholine in the process of transmission. He considered his findings as incompatible with the idea that acetylcholine metabolism is limited to synapses: "The acetylcholine metabolism is not a process which is specific for the synaptic junction." This conclusion, written in 1938, has been borne out by many biochemical and physiological data accumulated since then. The apparent inconsistencies and contradictions reported are readily explained in terms of the intracellular processes proposed.

Another aspect, which is frequently discussed in the literature, concerns the quantities of ester collected in the perfusate in relation to those required to produce an action. The first quantitative evaluation made by Dale and his associates revealed a discrepancy by a factor of about 100, in spite of the presence of eserine; this was considered by the investigators themselves as a difficulty for the mode of action proposed. Revised figures even increased the factor to 100,000. Recently, refined electrophoretic methods of application of acetylcholine to the motor end-plate were developed (Nastuk, 1953). With this efficient technique, it was found that about 6×10^6 molecules of acetylcholine produce a response at one motor end-plate of a frog sartorius muscle, although the time course of the response (15 msec.) is still not the same as that evoked by neural stimulation. This figure is of the same order of magnitude as that estimated by the writer 20 years ago on the basis of biochemical studies (Marnay and Nachmansohn, 1938; Nachmansohn, 1939a). The amount of ester which may be hydrolyzed at a single motor end-plate of frog sartorius muscle per millisecond is 3×10^9 molecules. This figure gives only the potential rate and not the amount actually metabolized, which could only be calculated if the excess of enzyme over the minimum required was known. In several unmyelin-

ated axons of invertebrates, the excess of enzyme was found to be about fivefold; in frog sciatic nerve, it appeared to be higher, but the results were less accurate, since the measurements were made at the limit of accuracy of the method used. On the other hand, the excess of enzyme may be higher at synaptic junctions than in axons. Moreover, the period of time required for the removal of the ester released per impulse is not known; this period may be either greater or—what is more likely—less than 1 msec. This introduces another factor of uncertainty. But the order or magnitude is comparable to the minimal amounts found to produce a response. On the basis of autoradiography of end-plates of a cat diaphragm treated with radioactive curare or decamethonium, Waser and Lüthi (1956) calculated that 8×10^6 molecules were fixed per motor end-plate. This is a slightly smaller figure, but still not very far from the values obtained with quite different methods and quite different types of muscle.

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Other discrepancies reported between effects of electrical stimulation and of acetylcholine applied may be attributable to the difference frequently encountered between the reactions of metabolic products released *within* a cell and those applied from the outside. The discovery of the appearance of acetylcholine in the perfusates was important because it was an indication that acetylcholine plays a role in transmission. However, even the much smaller discrepancy reported now, compared with that described earlier, fails to meet the principal objections raised above against using these findings for deciding on the mode of action of the ester. The original interpretation of the appearance of the ester has become untenable when it is considered in the light of all the physiochemical and biochemical data accumulated in favor of an intracellular process.

In this connection, the observations of miniature end-plate potentials (Fatt and Katz, 1952a) may be briefly mentioned. It appears likely that the action of acetylcholine is essential in these potentials. Their block by curare and their increase by esterase inhibitors supports such an assumption. These effects do not indicate, however, whether the release of acetylcholine is the primary cause, nor do they reveal the site of origin, since the ester may act in the same way when released on either side of the junction. Nerve terminals probably exert a strong influence upon the surrounding membrane of the effector cell. The events taking place, when a nerve ending makes a new contact with the muscle fiber or when it degenerates and disappears, may be considered as an indication. Various kinds of stimuli may pass all the time from the terminal, as long as it is functionally intact, to the opposite cell membrane. There may be an intermittent release of acetylcholine either within the pre- or the postsynaptic membrane, or in both. The interpretation of these potentials, the question of whether there is any relationship to the physiological events in conduction and to which particular event, is difficult. Their significance is made even more obscure by the finding that the potentials continue even in isotonic K_2SO_4-treated muscle, when the tissue is completely depolarized, the excitability of nerve and muscle has been abolished, and no Na^+ is present in the fluids; the character of the potentials (del Castillo and Katz, 1955b) is modified, however. At increased magnesium and lowered calcium concentration, the activity may also continue at an undiminished rate although the nerve impulse is no longer capable of initiating a response. Since these miniature potentials persist under

such extremely unphysiological conditions when excitation and transmission have become completely impossible, they cannot be linked directly to the physiological events taking place in transmission. That chemical reactions may go on and may have a stronger action than bioelectric currents, in other words, may produce effects when the currents fail to do so, is not surprising; chemical forces may be stronger and act more directly and efficiently than electric currents. This is especially to be expected in such complex and delicate structures as the neuromuscular junction, where geometry must play an important role in the response to electrical stimulation. This type of observation can certainly not be used as an argument that the membrane in physiological conditions is electrically inexcitable. In the same category belongs the reasoning based on the inability of some structures to respond to electric stimuli, whereas they react to chemical compounds. This applies particularly to the denervated electroplax of *Torpedo*. Marked alterations take place during degeneration of nerve terminals, as indicated by the extensive changes of structure. Failure of response to electric stimulation does not indicate that these structures under physiological conditions are electrically inexcitable.

Just as is the case with the appearance of acetylcholine in the perfusion fluid in activity, the existence of the miniature potential is not evidence for neurohumoral transmission. Neither of these two types of findings gives an indication and still less permits a decision as to the nature of the propagating agent in physiological activity.

VII. PRESENT STATE OF THE PROBLEM

Let us summarize the present state of the problem regarding the rôle of acetylcholine in synaptic and neuromuscular transmission. There is general agreement that acetylcholine plays an essential rôle in this process; the difference of opinion concerns the precise mechanism of action, the question whether acetylcholine acts in the pre- and postsynaptic membranes to generate currents as in conduction or whether acetylcholine acts as a neurohumoral transmitter in the original sense of the word.

To refer to the difference between axonal conduction and synaptic transmission as if the one is "electrical," and the other "chemical," is an unfortunate and misleading terminology; it veils the real issue. Some physiologists (Hodgkin, 1957; Eccles, 1957), referring to conduction as being "electrical," actually mean a purely physical process,

whereas in transmission they now accept the necessity of intervening chemical reactions. This is in contrast to previous views, e.g. those of Eccles (1946), in which transmission also was long conceived in "electrical," i.e. purely physical terms. In this view, the difference between conduction and transmission is, of course, basic in nature. The real issue under discussion may then be formulated as follows. Does conduction, also, require a chemical reaction for the generation of the electric currents and is acetylcholine essential for this process in all conducting membranes, or is the activity of acetylcholine specifically limited to the synaptic and neuromuscular junction, conduction being a purely physical process? Between these two views, the gap is very wide indeed. It can hardly be bridged. If, however, one accepts the essentiality of acetylcholine in both processes, the question remains, whether acetylcholine acts at synapses as a neurohumoral transmitter or whether it acts there too intramembraneously, as in the axon. This would obviously be a much less "fundamental" difference; it would be a minor matter of detailed mechanism. The writer does not find any convincing evidence for the originally proposed mode of action. On the other hand, the detailed mechanism still offers many problems in the axonal membrane, as well.

Nobody denies that the events at the synaptic junctions show marked differences in many respects from those observed in the axon. Whether these differences are "fundamental" is a matter of definition: fundamental in respect to what? In regard to macro- and microstructure, there are striking differences; in these instances the attribute "fundamental" may well describe the differences. These differences in structure must profoundly affect electric fields and electrical manifestations, time relations, response to various chemical agents, etc. But is the biochemical system responsible for the propagation of impulses in the elementary process on a molecular level different in the axon and at the synapse? If one categorically rejects the rôle of acetylcholine in the primary process of conduction, then of course it would be justified to speak of a fundamental difference on a molecular level, since in that view, conduction is purely physical, while transmission on the other hand is chemical. If one takes a less extreme view, it is more a matter of semantics, whether one considers the discrepancy between the two opinions as fundamental or not.

There are a number of general principles and notions resulting from advances in dynamic biochemistry which make it difficult, from a

biochemical point of view, to accept the mode of action proposed by the hypothesis of neurohumoral transmission. All reactions taking place in a living cell are chemically and energetically coupled. This coupling of enzyme catalyzed reactions is one of the most distinctive biochemical attributes of living matter. In contrast to processes in a homogeneous system, a condition of thermodynamic equilibrium does not exist, but rather a steady state. The coupling is very different from that observed in homogeneous enzyme solutions. Many biochemical reactions in an intact cell are structurally organized. Striking documentation is provided by the recent developments obtained by electron microscope studies in combination with enzyme chemistry (see e.g. Palade, 1956; Green, 1956). Even a process like oxidation, which is remote from more elementary activities of the cell, is not only localized in mitochondria, but is localized within the mitochondria in the cristae; the electron transfer proceeds there apparently in a well organized pattern. It seems difficult to assume that the fastest and most precise function developed by nature, the conduction of nerve impulses, includes at certain points chemical processes which are not structurally organized, not intramembraneous, and not even intracellular, but intercellular; that a substance produced in one cell acts upon a protein in another cell; that an uncoupling take place in the acetylcholine system, one of the fastest-acting systems known, so that part of the reaction, the formation and the release of acetylcholine, would be localized in one cell, while the reaction with the receptor and the hydrolysis by the esterase would occur in the other cell. Everything is possible, the living cell being a heretic, to use an expression of Frederic Gowland Hopkins, but to make it probable, substantial evidence must be presented for such a split of the system between two cells. The observations indicating that both acetylcholinesterase and receptors are present in nerve terminals make the assumption of an unorganized and split system difficult.

The notion of the biochemical unity of life has frequently promoted the development of biochemistry. Most important chemical cycles take place with only minor differences in primitive systems in a similar way as in all types of cells of higher animals. But their function and usefulness varies greatly with structural organization, etc. The interaction between ATP and actomyosin is today considered to be the basis of motility of all types of muscle throughout the animal kingdom. Since bioelectric currents and permeability changes have been shown to be inseparably associated with the acetylcholine system in the axon, which

is a much more favorable material for such studies than synaptic junctions because of its greater simplicity and accessibility, the difference of manifestations at the two sites of the same cell should be attributed *to different chemical forces or to a different molecular arrangement of* a chemical system only when really strong experimental evidence is available. Nobody will deny that at the synapse there may be quite a few additional secondary factors of importance modifying the events at junctions. Very little is known about such factors. Del Castillo and Katz (1956) make the statement: "Inferences about the physical state and chemical properties of nerve endings have had to be made by indirect argument and from analogies; and are therefore likely to be at fault." If one agrees with that statement, as the writer does, it appears difficult to accept the conclusion that the hypothesis of neurohumoral *transmission is already firmly established.*

Dale, in his Harvey Lecture (1937), envisaged the possibility of a much wider rôle of acetylcholine than that proposed at that time: "If the liberation of a chemical mediator at a nerve ending should prove to be not a process peculiar and limited to that ending but merely a local intensification, to ensure transmission to a contiguous cell, of a process which actually figures in the propagation of the impulse along the nerve fiber, we should have to make yet a further revision of our existing conceptions. Some minds have undoubtedly felt difficulty in postulating a complete breach in the nature of the processes concerned in transmission, where the excitation passes from nerve ending to effector cell. This particular difficulty would then disappear but only at the cost of a more fundamental change of conception concerning the nature of the propagated wave of excitation than any which has yet been seriously considered." This intuitive statement is all the more remarkable since at that time there was no experimental basis and hardly even any indication for such a bold and imaginative assumption. It is impressive that at a symposium on neurohumoral transmission in 1953, Dale, one of the great pioneers in that field, recalled his statement of 1937 and warned the audience not to consider the theory of neurohumoral transmission as being a final solution of the problem (Dale, 1954). This spirit and attitude is certainly in striking contrast to the categorical statements of some of the adherents of his theory. Dale is reluctant to accept the unified theory; he considers it as a simplification which might lead us astray in biology. Methods of approach to the problem must of necessity greatly affect our thinking. The

remarkable unity of biochemical principles and systems throughout the great diversity of living cells must influence the reasoning of the investigator attacking the problem primarily by chemical analysis. Physiology and pharmacology, on the other hand, study manifestations of intact cells, cells characterized by a nearly infinite variety as to organization and structure. It may be useful to recall the words of J. J. Thomson, in his book, "The Corpuscular Theory of Matter," that "the object of a theory is to connect or coordinate apparently diverse phenomena." It is on the molecular level that morphology, physiology, and biochemistry must eventually meet. Considering the present state of knowledge of events in conduction and transmission, it seems to the writer that there is at present no compelling reason to attribute the differences to either a modification of the molecular organization or the functional significance of the acetylcholine system; it seems to him rather more appropriate to adhere to one of the fundamental rules of scientific thinking, not to assume two different principles without necessity.

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CHAPTER VI

Some Aspects of the Biophysics of Muscle

ROBERT W. RAMSEY

I. INTRODUCTION

With one major exception, this chapter is limited to a discussion of some biophysical aspects of vertebrate skeletal muscle, chiefly that of the frog. The exception is a brief description of the excitatory process in squid nerve which serves to emphasize some of the problems still to be solved in understanding the excitatory process in muscle. Space, personal preference, and previous knowledge of the topics to be presented elsewhere in this volume dictated both the narrowness of scope and the selection of topics discussed here.

No attempt has been made to cite all of the relatively recent reviews and monographs which include some of the topics discussed. Those cited were not chosen by caprice, however; they simply reflect the author's view that they are clear expositions of a particular topic. While the provincialism of this article is to be deplored, the author wishes to plead that the time that could be allotted to the preparation of this chapter prevented reading and rereading the many scientific reports published in languages other than English.

II. FACTORS CONCERNED WITH EXCITATION

A. EXCITATION—A GENERAL DESCRIPTION

The study of the excitable properties of tissues has had a long and interesting background (Lapicque, 1926; Monnier, 1934; Rashevsky,

1938; Katz, 1939a; Schaefer, 1942). The main features of the excitatory process with respect to any stimulus were discovered many years ago. The great majority of investigations have concentrated on using an electrical stimulus for investigating the excitable properties of tissues. For direct currents, this led to the strength-duration curve, and virtually all efforts until about fifteen years ago were concentrated on endeavoring to set up an electrical analog that would account for the strength-duration curve and other phenomena of excitation. All such attempts, sooner or later, either ran into inconsistencies or else were so complicated and had so many arbitrary constants that the equations derived could be made to fit anything.

Blair (1932) tried a different approach. He sought to describe the general phenomena of excitation in terms of equations that would not lead to inconsistencies, without specifying what the underlying physical mechanism was. He proposed that upon application of a direct current to muscle tissue an undefined "state of excitation" was built up at the cathode, proportional to the current times the duration of the stimulus, and if the stimulus was adequate this buildup of excitatory state E *reached a threshold value* E_0 *which initiated the propagated action* potential. Initially, this threshold for muscle will be assumed to remain constant. In nerve, another slower process is involved; the threshold increases at a rate proportional to the shock strength. This latter phenomenon, which was shown by Rashevsky (1933) to have analogous kinetics to the growth of the excitatory state, is now universally called accommodation, after the terminology of Hill (1936), who used essentially the same kinetics as Rashevsky. A discussion of accommodation will be omitted here because if it occurs to any extent in muscle it is probably in response to injury. (In partially injured muscle fibers, break excitation at the anode is occasionally seen and this is interpretable in terms of a simultaneous negative accommodation and excitatory [diminished] process at the anode.)

Another phenomenon studied for many years was that known variously as the summation of inadequate stimuli (inadequate in the sense of duration, not voltage) or latent addition. If a direct current stimulus adequate with respect to voltage, but inadequate with respect to duration, was applied and removed, the state of excitation built up by the first shock did not disappear instantaneously but decayed at a rate proportional to its own magnitude.

Older hypotheses had suggested that when a stimulus was applied

to an excitable cell, some process occurred which tended to oppose the buildup of a state of excitation. Nerst, for example, suggested that the excitatory state was a local accumulation of ions at the membrane which was opposed by back diffusion. Blair (1932) suggested that the state of excitation that was built up at a rate proportional to stimulus strength was opposed by a process whose rate was proportional to the state of excitation built up. The net rate of accumulation of excitatory state, therefore, was given by equation (1).

$$\text{Net rate} = KV - kE \tag{1}$$

V = voltage (the resistance of the circuit being constant); K = a proportionality factor to allow for the fact that some of the current is ineffective due to shunting; E = state of excitation built up at any time t; k = constant whose value equals the rate of decay when E equals 1.0.

The integral of equation (1) is shown in equation (2).

$$E = \frac{KV}{R}(1 - e^{-kt}) \tag{2}$$

For a just sufficient stimulus $E = E_o$ and for muscle this may be taken as constant-equation (3).

$$E_o = \frac{KV}{k}(1 - e^{-kt}) \tag{3}$$

For the longest effective stimulus, e^{-kt} is negligible, therefore V must have at least the value kE_o/K in order to excite. By definition, this minimal value is equal to the rheobase (R). Substituting R for kE_o/K in equation (3) we arrive at equation (4).

$$R = V(1 - e^{-kt}) \tag{4}$$

Equation (4) represents the ordinary direct current strength-duration curve for muscle. It and the corresponding growth of the excitatory state are diagrammed in Fig. 1. The equation can be conveniently tested by putting it in the form shown in equation (5).

$$\log_e \frac{V}{V-R} = kt \tag{5}$$

If the left hand member of the equation is plotted against t a straight line having the slope k is obtained.

Several other useful consequences of this equation have been noted by Blair. Lapicque defined the term chronaxie as that time required for a stimulus of twice rheobasic strength to excite. Substituting $2R$ for V in equation (5) shows that the chronaxie $= 1/k \log_e 2$. Since $1/k$ is the time constant in the usual sense, chronaxie is a measure of the time

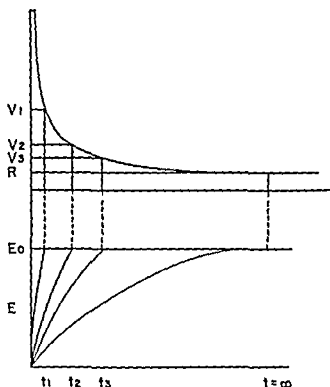


FIG. 1. Direct current strength-duration curve (above) and corresponding growth of the excitatory state E (below) according to equation (4). Blair (1941) published a similar diagram.

constant of the decay process. Also if the stimuli are made very brief, equation (4) reduces to Vt = constant $= R/k$ so that $1/k$ or the reciprocally related chronaxie can be evaluated if R is known.

If condenser shocks are used instead of constant current stimuli, Blair (1936) has shown that the equation corresponding to equation (5) is equation (6).

$$cr \log \frac{V}{R} = 1/k \left(\log \frac{V}{R} + \log cr\right) + \frac{\log k}{k} \qquad (6)$$

R is the direct current rheobase, C the capacity of the condenser, and r the resistance of the total circuit.

Returning to equation (5) it has been found that it is rarely obeyed exactly. The simpler the preparation used, (a single muscle fiber) the less is it obeyed. Blair (1941), in a very thorough analysis, demonstrated that failure to obey equation (5) was not due to failure of the two basic postulates respecting the rates of growth and decay of the excitatory state but rather was due to the fact that the current across the excitable membrane was not constant but was distorted for very short shocks.

Chronaxie admittedly is a poor measure of membrane properties. It varies widely for the same tissue with different experimental conditions. The alpha excitability of muscle which is measured by stimulating with large fluid electrodes (Lucas, 1907), may be expected to more nearly parallel alteration of membrane properties, but even in this instance interpretation is very complex. In some quarters, it is fashionable, therefore, today, to ignore any such general description of excitation. Thus some justification for its inclusion here perhaps is called for. In company with analogous kinetics for accommodation, it must be admitted by all that it accounts in a semi-quantitative way for a very wide range of phenomena in excitation and conduction in both nerve and muscle. Its utility therefore in screening drugs for their effects on these phenomena is very real, though admittedly not very informative as to their ultimate mode of action. Its value in a second realm is in teaching. Put into words and diagrams, the simple kinetics involved can account for most of the phenomena observed in excitation and conduction in a manner the average medical student can understand and use. Even today, the main features of the exact physical description of excitation and conduction have only been elucidated for the giant axons of the squid, so that this more general description appears completely warranted. To paraphrase Cole (1955), we may be amazed that the one and two factor theories have such a complicated basis but we may also be more tolerant of their deficiencies. In fact, I cannot help but be amused that my good friend Dr. Cole now has gone around the clock and admits that empirical equations involving this time "a dozen analytical functions and more than as many constants" might have some utility. For my part, the two factor hypothesis also still has *utility*.

B. The Resting Membrane Potential

The analyses of Katz (1896) established the fact that the intracellular cation content of muscle tissue was predominantly potassium and that its chloride content was low and might be zero. Bersntein (1902) showed

that if the muscle membrane was permeable to potassium and impermeable to other cations or the intracellular anions, such a system should give rise to a potential difference across the membrane whose magnitude would be given by the Nernst equation or equation (7).

$$E = \frac{RT}{F} \ln \frac{K_i^+}{K_o^+} \tag{7}$$

R, T, and F have their usual significance and the subscripts i and o refer to inside and outside of the cell, respectively. Such a system would be in thermodynamic equilibrium when the outward force of diffusion was just balanced by the inwardly directed force of electrostatic attraction E.

In the early work on resting potentials, a direct test of equation (7) was virtually impossible. It was established with reasonable certainty that little of the potassium was bound and that probably the concentration of potassium could be taken as a measure of its activity. The measured potentials which were obtained by measuring the potential difference between an uninjured surface of a muscle and its crushed end (or one dipped in isotonic KCl) were always much lower than the theoretical. The failure to record the theoretical potential difference could quite reasonably be ascribed to shunting, potentials arising at the injured end, and junctional potentials. If the potassium content of the fluid bathing the uninjured surface of such a preparation was increased the potential difference fell in a manner roughly similar to that which would be predicted by equation (7). Similarly, the effect of temperature was explainable in terms of the Nernst equation. The most pertinent of this early work is discussed by Fenn (1936) and also by Davson (1952) and Hodgkin (1951).

Boyle and Conway (1941) showed that muscle was also permeable to chloride. On the assumption of sodium impermeability they suggested that the distribution of ions followed a Donnan membrane equilibrium. For high concentrations of potassium in the bathing medium, their data supported the concept that the distribution of ions was in accordance with a Donnan membrane equilibrium but in the physiological range of potassium concentration it was not. With the development of intracellular recording of muscle membrane potentials by Graham and Gerard (1946), later refined by Ling and Gerard (1949a), the resting membrane potential at 20°C. was found to be close to the theoretical value of 99 mv. predicted from equation (7), if the analytical values of 126 mM K per kilogram muscle water and 2.5 mM

K per kilogram plasma from Boyle and Conway's figures are substituted in equation (7).

In studies with radiactive sodium as a tracer, Levi and Ussing (1948) showed in washout studies of a muscle previously equilibrated in Ringer containing radioactive sodium that there was an initial rapid washout followed by a much slower appearance of radioactive sodium. They interpreted the initial rapid washout as loss of Na^{24} from extra-cellular spaces and the later slower washout as loss of Na^{24} from intra-cellular spaces. The earlier work of Heppel (1940) on K deficient rats also indicated that the muscle membrane is permeable to sodium. The acceptance of the idea of sodium permeability necessitated a revision of Boyle and Conway's (1941) Donnan membrane hypothesis. In general, this has been approached in two ways. One scheme proposes that there is a metabolic sodium extrusion pump (Dean, 1941). Another is the idea of "exchange diffusion" (Ussing, 1949) wherein a carrier in the membrane must always have a sodium ion attached to it. This carrier could shuttle between inner and outer edges of the membrane exchanging its sodium ion and thus account for the permeability of sodium, but the overall sodium content would not increase. This mechanism will not account for Heppel's results or for the known extrusion of sodium when K-deficient animals are restored to a normal diet, but it has the merit of not being an active transport mechanism.

The Nernst equation would only be valid under the following conditions: (a) the membrane itself possessed no charge; (b) the membrane was solely permeable to potassium; and (c) the system was in a steady state. We know now that the membrane is permeable to sodium and chloride as well as potassium and that there is no steady state but rather a continuous flux of all three ions. Further, there is little doubt that the membrane itself is charged in the living cell. Whether this charge arises as a consequence of the ion distribution or causes the ion distribution will be discussed briefly later. At all events, as Teorell (1936) has emphasized, in such a system there is a Donnan potential between the exterior of the membrane and the exterior solution and another Donnan potential between the cytoplasm and the inner part of the membrane, as well as a membrane potential. Therefore, the total potential difference from interior to exterior is the sum of these three. In general, it is impossible to set up any theoretical equation for the potential difference without a precise knowledge of how each ion is transported in the membrane and the relative ion permeabilities. One highly empirical

mechanism that will account for the facts of ion transport in squid nerve has been postulated (Hodgkin and Huxley, 1952) but comparable data on muscle that would permit a similar development does not exist.

Neglecting the surface potential differences and assuming that the electric field in the membrane is constant (a very unlikely assumption [Teorell, 1936]), Goldman (1943) solved the diffusion equation for univalent electrolytes and applied it to biochemical data then available. With the same assumption Hodgkin and Katz (1949) applied the equation (8) to the squid nerve data.

$$E = \frac{RT}{F} \ln \frac{\{P_k [K^+]_i + P_{Na} [Na^+]_i + P_{Cl} [Cl^-]_o\}}{\{P_k [K^+]_o + P_{Na} [Na^+]_o + P_{Cl} [Cl^-]_i\}} \quad (8)$$

The permeability coefficients are shown in equation (9).

$$P_i = \frac{RT}{Fd} U_i b_i \quad (9)$$

U_i is the mobility of the ion in the membrane, b_i the partition coefficient for the ion between membrane and solution, and d the thickness of the membrane. Jenerick (1953) determined the permeability ratios P_{Na}/P_K and P_{Cl}/P_K by solving two simultaneous equations set up by substitution of Boyle and Conway's (1941) figures for the internal ion concentrations in one equation and the measured intracellular recorded membrane potentials for two different external concentrations of KCl in the other. Using the values so obtained, he tested equation (8) by measuring the membrane potentials at different externally applied KCl concentrations. Except for the unexpectedly high value of membrane potential observed when the potassium concentration in the medium is zero, equation (8) fitted the data very well (Fig. 2). At least it predicts a finite value when the exterior potassium is zero instead of the infinite value required by the Nernst equation. It is well to bear in mind that the assumption involved in the above test of equation (8) is that ions move through the membrane solely under the influence of their electrochemical gradient and that no active transport is involved. Steinbach (1952) has presented evidence that uptake of potassium in muscle depends on sodium extrusion. Keynes and Maisel (1954) found no reduction in sodium efflux after muscle had been doubly poisoned with cyanide and iodoacetate for several hours at room temperature. Hodgkin and Keynes (1955) in the giant axons of cephalopods find a loosely coupled metabolic relation between po-

tassium influx and sodium efflux for at least part of the potassium present in nerve. Jenerick's assumption that the intracellular ionic content did not change appreciably with the altered KCl content of the external medium within the time required for the potential measurements may or may not be valid.

The fact that Ling and Woodbury (1949) found that the membrane potential is proportional to the absolute temperature, as equations (7) or (8) demand, is probably not a very good test since over a 30°C. temperature range the theoretical potential difference would only vary about 10 mv. Further, according to Harris (1952), the activity co-

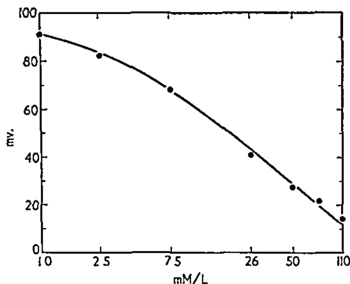


FIG. 2. Test of equation (8). Ordinates: membrane potentials of a muscle fiber; abscissa: external potassium concentration in mM/L. Taken from Jenerick (1953), 438, Fig. 5.

efficient of some of the potassium changes at 0°C., as judged by the fact that about 20% of muscle K^+ exchanges much more slowly than the rest. This is in contrast to the exchange at 18°C., where all (within 5%) of the K^{42} used exchanged exponentially. On the basis that at 0°C., 20% of the K^+ is bound, and using values of the concentrations of potassium in and out, as published in the literature, he compares the calculated values of membrane potential using equation (7) with those measured by Ling and Gerard (1949a) and finds them in agreement. There are other indications that various fractions of potassium have different activities (Harris, 1956).

Test of equations similar to equation (8) can be done by altering the internal ion concentrations instead of the external concentration. Falk

and Gerard (1954) injected KCl and NaCl solutions into a single muscle fiber *in amounts that should have led to readily detectable* changes in the membrane potential as calculated from equation (8), but they did not observe any significant change. They suggested that a sodium pump term having an electrical sign be added to equation (8). If this added term were responsible for approximately one-third the membrane potential, they felt their injection experiments could be accounted for partly in terms of equation (8). Injection experiments into the giant axons of squid by Grundfest (1955) similarly did not lead to the expected change of membrane potential.

Shaw *et al.* (1956) took advantage of the fact that the muscles of the toad *Bufo marinus* undergo large changes in potassium and sodium content with the season of the year. Intracellular recording of membrane potentials of fibers of muscles *in vivo* gave constant membrane potentials, even though the potassium content of different muscles varied from 72 to 132 mM per kilogram wet weight and the sodium varied from 68 to 10.1 mM per kilogram wet weight. While extracellular space was not measured, their argument that much of these ion shifts were intracellular seems reasonable. The paired muscles were equilibrated in Ringer solution and their potassium and sodium contents shifted so that the membrane potential was in accordance with the Nernst equation. In muscles whose sodium content was increased by prolonged soaking in cold Ringer solution containing little potassium, Desmedt (1953) found that the resting and action potentials varied as predicted by classic theory.

In ryanodine contractures, there may be very large shifts in water and electrolytes generally resulting in a large net loss, (Ramsey *et al.* 1952 b) but according to Jenerick and Gerard (1953) there may be little or no change in membrane polarization.

It has long been known that interference with the metabolism of muscle lowers the membrane potential. Ling and Gerard (1949b) found that the membrane potential could be divided into two fractions, A and B. The membrane potential is relatively insensitive to anoxia but in combination with iodoacetate the A fraction (25 mv.) was reduced and was much more sensitive than the B fraction (55 mv.). Procedures which reduced the creatine phosphate content of muscle caused a corresponding reduction in the A fraction. ATP injected into single muscle fibers had no effect on membrane potential or the contractile mchanism. If applied to the outside of a fiber, it caused a series

of action potentials, which suggested ATP may be involved in conduction (Falk and Gerard, 1954). When the metabolism of the muscle was increased by stretching it, the membrane potential was much more sensitive to metabolic poisons than in the unstretched condition, but the stretch per se had no influence on the membrane potentials (Ling and Gerard, 1949c). They also found (Ling and Gerard, 1949a) that the resting potential was the same over a range of pH from 5 to 10. Harris (1956) has suggested that the resting potential may be partly a Donnan potential due to large phosphate ester anions (the A fraction) and the remainder a diffusion potential. As he emphasizes, a better test of equation (8) would be to measure the permeability ratios and compare them with those predicted on the basis of equation (8).

Direct measurement of the permeabilities of potassium, sodium, and chloride in muscle was always admittedly very difficult, and they are rendered much more so by the discovery that evaluation of the total flux from studies of tracer flux may not always be valid. Since Harris (1956) thoroughly reviews this whole field it will not be gone into here.

1. *Summary*

An extensive mathematical treatment applicable to membrane potentials and based on considerations similar to those used in deriving equation (8) has been developed by Johnson *et al.* (1954). Neglecting for the moment other pertinent evidence, the fundamental assumption involved in the derivation of equation (8) was that each ion only moved under the influence of thermal and electrical forces. Its chances of crossing the membrane, in other words, depended solely on whether it hit it or not and on the sign of the electrical gradient and did not depend on whether a like ion had just preceded it through the same space. In this sense it was independent. The recent investigations of ion fluxes in nerve and muscle throw considerable doubt on the correctness of this assumption. In frog muscle, for example, Keynes (1954) finds that only about one-tenth of the total membrane conductance can be accounted for if it is calculated from the potassium flux, though on other grounds K^+ ions might be expected to carry about half the current.

From the evidence available at this time, it does not seem possible to explain the origin and magnitude of the membrane potential in terms of one mechanism. There may be parallel generators of bioelectric potential such as Grundfest (1955) and others have discussed. Under

some conditions, they may be considerably out of phase. Like two slightly out of phase alternating current generators thrown simultaneously into a line, they proceed to "hunt" until both are in phase again.

C. The Dominant Physical Events in Excitation

The sequence of events taking place during the course of excitation is very much better known for the membrane of the squid giant axon than it is for any other tissue (Cole, 1955; Hodgkin and Huxley, 1952). While there are both quantitative and qualitative differences in the excitable properties of muscle membranes as compared with squid

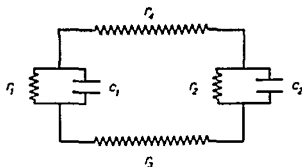


Fig. 3. Equivalent submarine cable circuit. r_1, r_2, c_1, and c_2 are the resistances and capacities of the membrane at the regions of current exit and extrance, respectively; r_3 is the resistance of the core and r_4 is the resistance of the medium.

giant axon membrane or for that matter with membranes of different muscles (Weidmann, 1957; Hecht, 1957; and others), the similarities are sufficiently great to warrant a short description of the chief developments that led to our present day understanding of the physical events involved in excitation of the squid axon.

1. *Cable-Like Behavior and the Local Response*

The early studies of electrotonic potentials suggested that nerve and muscle behaved like a submarine cable, that is, it had a conducting core surrounded by a semi-insulating sheath with a rather large capacity. Electrically, this would be represented as a capacity shunted by a resistance. The relatively slow buildup of electrotonic potential could be explained by assuming that the nerve or muscle could be represented by an equivalent circuit consisting of many such units connected in parallel between the internal resistance of the core and the external resistance of the medium as in Fig. 3. If a small rectangular shock was applied to such a system, it would become smaller and longer and

travel more slowly down the cable as the partially charged condensers discharged through their shunted resistors and the whole pulse would decrement exponentially to zero over a very short distance of travel. The sign of the potential changes at the cathode would be the mirror image of the corresponding changes at the anode. For a long time, it was believed that when the catelectrotonus reached a certain critical threshold, the membrane suddenly became highly permeable to all ions at the cathode, with the result that the low resistance permitted

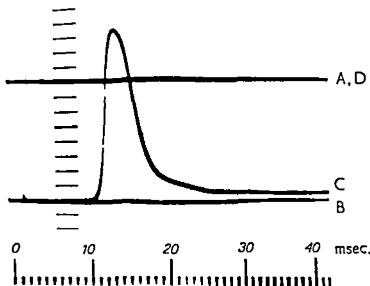


FIG. 4. Resting and action potential of a muscle fiber recorded at 6°C. External diameter of electrode tip, 0.5 μ; resistance of microelectrode, 57 MΩ. Ordinate scale, 10 mv. steps. Abscissa, msec. Records A and D were obtained with the microelectrode outside the fiber at the beginning and end of the experiment; B and C were obtained with it inside in the resting and stimulated conditions. From Nastuk and Hodgkin (1950), p. 48, Fig. 4.

current flow from the adjacent charged membrane, with consequent depolarization and hence propagation of the impulse.

Hodgkin (1938) showed that this view was incorrect; for very small currents of short duration, the resulting electrical changes at the cathode and anode were purely electrotonic, but for slightly greater pulses, still subthreshold, a local potential change at the cathode resulted which was far greater than could be accounted for by electrotonic effects as judged by the magnitude of the electrotonic effect at the anode. For slightly larger stimuli, the local change at the cathode increased very greatly until for a threshold shock it set off the propagated spike of the action potential. He also showed that the shock strength

influenced the latency for the development of the spike. In all of the earlier work, it was assumed that the maximum potential change of the spike could never exceed the resting membrane potential. Since this was always measured by external electrodes, there was no good way of testing whether this assumption was correct or not.

2. *Ion Fluxes in Excitation of Squid Nerve*

In 1939–40 the big break in this field occurred, which with subsequent developments virtually revolutionized our thinking in the study of the excitatory process. Almost simultaneously, Hodgkin and Huxley (1939) and Curtis and Cole (1940) placed an electrode intracellularly in the giant axon of the squid and recorded the action potentials directly. To the astonishment of all, the magnitude of the action potential was far greater than the resting potential, the overshoot of the spike making the inside of the cell positive to the outside. With the development of intracellular recording for muscle by Graham and Gerard (1946), similar overshoots were found in muscle (Fig. 4). Hodgkin and Katz (1949) found that the magnitude of the action potential was proportional to the logarithm of the external sodium ion concentration. Whereas formerly only potassium fluxes were considered, now sodium fluxes had to be taken into account as well.

Study of these ion currents was virtually impossible until what has been called the "voltage clamp" technique was developed by Marmont (1949) and Cole (1949). Briefly, this consists of threading a nerve through two external guard electrodes on either side of a center external measuring electrode. A long electrode, spanning guard and center electrodes, are inserted intracellularly in the axon so that the membrane potential and current density are the same at all points in the central region, thus preventing propagation of the impulse. Since the total current across the membrane is the sum of the capacity current and ion current, it was necessary to eliminate the capacity current in order to study the ion currents. This was done by imposing a stepwise potential difference across the membrane, which by a feedback system was maintained constant. With this "voltage clamp" imposed on the system all current flow is ionic, except for an initial capacitive transient. In a series of beautiful experiments summarized by Hodgkin and Huxley (1952), the ionic currents were analyzed as to the part played by sodium, potassium, and "leakage" fluxes. Since the "leakage" flux due to other ions was small, only the sodium and potassium fluxes will be dis-

cussed here. If the membrane potential was suddenly lowered by approximately 10 mv. or more, following the quick capacitive transient, the resulting current flow was inwardly directed, that is, opposite to that which would have been caused in an ohmic resistance for the same voltage change. When the membrane potential was lowered by a threshold amount, this inwardly directed current due to sodium ions was sufficient to account for the charging of the membrane capacity during the rising phase of the spike. Later, this was succeeded by an outwardly directed potassium current which restored the membrane potential. The driving force of each of these ionic currents can be represented by the product of a conductance (permeability coefficient) G and an electrical potential difference represented as the difference between the membrane potential Em and the electrochemical potential of the ion in question, as given by the Nernst equation or equations (10a) (10b).

$$I_{Na} = G_{Na} (Em - E_{Na}) \quad (10a)$$

$$I_{K} = G_{K} (Em - E_{K}) \quad (10b)$$

Both conductances are dependent on membrane potential (but not on current) and on time.

With the lowering of membrane potential by a stimulus there is first an inrush of sodium current lowering the potential still further with a rapid increase in membrane conductance. As a consequence, the inwardly directed sodium current gives rise to the rapid rise of the spike which includes the overshoot, the total potential being equal to the sodium potential. When the peak of the action potential is reached an inactivation process sets in, lowering sodium conductance. Potassium conductance lags behind that of sodium but increases and along with inactivation of sodium conductance restores the membrane potential in the falling phase of the spike. Hodgkin and Huxley (1952) have discussed a possible mechanism as to the manner in which the membrane could work, but it is so empirical it will not be given here. Thus, r_1 of Fig. 3 may be symbolized by Fig. 5, taken from Cole (1957), where E_K and E_{Na} are the Nernst potentials of potassium and sodium, and "Kal" and "Nat" are the conductance controls which the membrane potential varies both with respect to speed and extent. As Cole (1957) points out "Kal" and "Nat" are the essence of the membrane properties but since they can only be derived from potential step data they are unavailable for any other tissue. Hodgkin and Keynes (1955) found

that metabolic poisons such as dinitrophenol, cyanide and azide influenced these control systems in recovery in that sodium extrusion and potassium influx were markedly reduced. Sodium influx and potassium efflux were unaffected. Thus, conduction of the impulse was unaffected, but presumptively if one could give enough stimuli both E_{Na} and E_K could run down and conduction would no longer be possible. In this respect, muscle appears to be quite different, since Keynes and Maisel (1954) report that they found no difference in sodium efflux in muscle doubly poisoned with cyanide and iodoacetate.

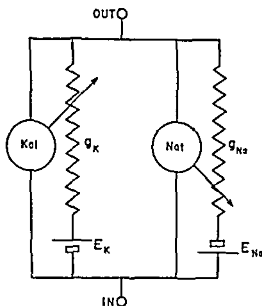


FIG. 5. Equivalent system for r_i of Fig. 3. G_K and G_{Na} conductance of potassium and sodium across membrane; Kal and Nat membrane conductance controls; E_K and E_{Na} the Nernst potentials of potassium and sodium, respectively. From Cole (1957), p. 660, Fig. 3.

3. *Electrotonic Responses of Muscle to a Subthreshold Shock*

Rushton (1937) showed that if a small constant current was applied to an excitable tissue whose equivalent electrical analog was similar to that of Fig. 3, the resulting electrotonic potential V_o generated under the poles of the battery should decrement exponentially in the extrapolar regions according to the equation (11).

$$V = V_o \exp\left(\frac{-X}{\sqrt{a}}\right) \tag{11}$$

where X is the distance and:

$$\sqrt{a} = \sqrt{\frac{rm}{r_i + r_o}} \tag{12}$$

rm = transverse resistance times unit length of the fiber, r_i is the internal resistance of the fiber per unit length, and r_o is the resistance of

the outside fluid per unit length. This factor $\sqrt{a}$ has been called characteristic length or space constant λ and is an index of electrotonic spread. This relationship is only true provided r_i in Fig. 3 remains constant. Actually, a polarizing current alters the resistance, increasing it at the anode and decreasing it at the cathode. This rectification is reversed if phosphate is omitted from the medium (Jenerick, 1953). In effect, the electrotonic extrapolar decrement will no longer be strictly exponential.

Katz (1948) measured the electrotonic potentials developed in response to an applied current on small bundles of frog muscle fibers and found that the decrement is essentially exponential, as predicted from theory, λ having values between 0.47 and 1.15 mm. From data obtained by measuring the appropriate terms with an intracellular electrode, Jenerick (1953) has calculated that the space constant λ should diminish from a value of 2.40 mm. to 0.41 mm. when the membrane potential is lowered from 105 mv. to 17 mv. From similar measurements using an intracellular electrode, Nichols (1956) calculated that λ increased in denervated but not atrophied frog muscle fibers. The specific membrane resistance in these latter preparations was more than double (10,300 Ω cm.$^{-1}$) the normal value but the capacity remained unchanged. As a consequence, λ was increased and the rise and fall of the electrotonic potential was slower. The resting and action potential were unchanged. In similar preparations, Harris and Nichols (1956) found that the membrane permeability to potassium was reduced. These findings taken together were considered as evidence that the resting membrane conductivity is mainly determined by potassium permeability. In a comparison of potassium fluxes and membrane conductance in frog muscle, however, Keynes (1954) claims that only about one tenth of the total membrane conductance can be accounted for if it is calculated from potassium flux. Since, on other grounds, much more of the membrane conductance would be expected to be due to potassium flux, Keynes argues that the movement of cations across the membrane is not completely independent of another ion traversing the membrane just previously at the same point.

4. *The Local Response of Muscle at the Cathode*

Subliminal currents produce a local response at the cathode (Kuffler, 1942), particularly if their magnitude is one-third or more the threshold value (Katz, 1948). Fig. 6 shows for different current strengths the essentially ohmic electrotonic potentials at the anode in contrast to

the buildup of local response at the cathode. It will be noted that in all of its main features the buildup of the local response is analogous to that in nerve. The rate of rise is more rapid, the greater the strength of the polarizing current, and if this is large enough, the local response sets off the spike of the action potential. For polarizing currents of less strength than depicted in Fig. 6, Katz (1948) showed that the potentials at the cathode were essentially electrotonic.

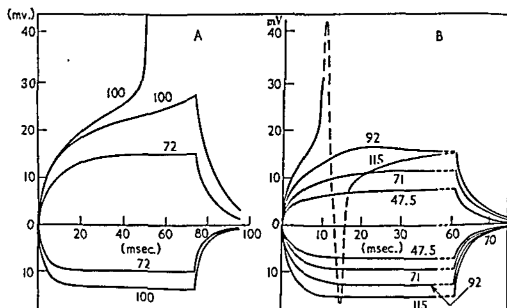


FIG. 6. Superimposed records of potential changes at the polarizing electrode. Cathodic potentials shown as upward deflections. Relative current strength shown in the figure. A and B, two experiments on bundles of four fibers. From Katz (1948), p. 514, Fig. 3.

5. *The Action Potential of Muscle as Recorded by External Electrodes*

In respect to action potentials, whole muscle behaves like a volume conductor (Craib, 1928; Wilson *et al.*, 1933). Referring to Fig. 7, wherein for simplicity a single cell in Ringer solution is diagrammed as having just been excited at its left hand end, the potential of the point P is equal to $V = \varphi\, \omega$ where φ is the electric moment per unit area of membrane and ω is the solid angle subtended by the membrane at P. To a first approximation this solid angle will be given by equation (13).

$$\omega = A \frac{\cos \theta}{r^2} \tag{13}$$

A equals the area of the depolarized disc, r equals the length of line

drawn between P and the center of the disc, and θ equals the angle between r and the axis of the disc. The potential V of P will therefore be given approximately by equation (14).

$$V = \varphi A \frac{\cos \theta}{r^2} \tag{14}$$

As Wilson points out, this is equivalent to assuming that all of the

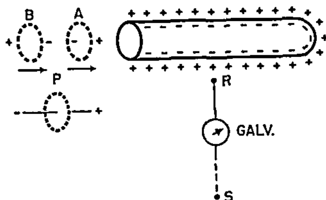


FIG. 7. Representation of depolarization as an equivalent dipole A in a volume conductor and repolarization as a dipole B with its sign reversed. For explanation and credit c.f. p. 22 and sequence.

positive charges of the disc are concentrated into one positive charge at its center on the positive side, and that all negative charges are concentrated into one negative charge at its center on the negative side. The excited cell therefore is equivalent to a dipole oriented as indicated at A and moving to the right. If recovery begins at the point first excited at the left, the repolarization can be represented as a dipole oriented in the reverse direction moving to the right, as shown in B.

If the potential difference is recorded between electrode R and some far distant electrode S which stays sensibly zero as the excitation dipole approaches R, R will gradually swing positive to a maximum value and then abruptly swing maximally negative. In a cardiac strip in which the whole tissue is excited before recovery begins, R would gradually become less and less negative, returning to zero potential as the last of the strip was excited (Blair *et al.*, 1941). For tissues such as skeletal muscle with a short refractory period and slow conduction velocity, the recovery dipole follows so quickly on the excitation dipole that the resulting complex would be expected to be triphasic (Craib, 1928). When to this complication is added some degree of asynchrony

in conduction of different fibers of a muscle and also the artifact of movement, the resulting action potentials can become very complex and difficult to interpret. While in simple cable theory the true monophasic action potential can be calculated from records obtained with external electrodes, in practice it rarely has been done.

6. *The Action Potential of Muscle Recorded with an Intracellular Electrode*

The electrode developed by Graham and Gerard (1946) and later improved by Ling and Gerard (1949a) for the first time permitted an accurate measure of the magnitude and time course of the action potential in both skeletal muscle fibers and heart syncytial fibers (in this latter case the Ling-Gerard electrode was modified by Woodbury and Brady (1956)). These electrodes have now been used by many workers and the variation found in the magnitude of both resting potential and action potential for the same type of tissue under similar conditions is astonishingly small. In every study, the finding is that the action potential "overshoots" the resting potential making the inside of the fiber positive to the outside by approximately 30 mv. for frog skeletal muscle at 20°C.

With a resting membrane potential of 90 mv. the spike of the action potential is about 120 mv., followed by a rather prolonged negative after potential (Fig. 4). According to Jenerick and Gerard (1953), the spike is initiated whenever the normal membrane potential is suddenly lowered to about 55 mv., a lowering of about 35 mv. In nerve, this critical depolarization is of the order of 10 mv.; the greater depolarization required for muscle is probably a consequence of the fact that the muscle membrane has a much greater capacity per unit area than nerve.

Nastuck and Hodgkin (1950) showed that the magnitude of the action potential increased linearly with the logarithm of the extracellular sodium ion concentration as it should if the rising phase of the action potential is due to an inwardly directed sodium current whose magnitude is a function of the difference between the membrane potential and the Nernst potential of sodium. Desmedt (1953) found that the action potential decreased as intracellular sodium increased and that the rate of rise of the action potential is proportional to extracellular sodium concentration, being little affected by intracellular sodium content, whereas the maximum rate of fall of the action potential is proportional to intracellular potassium concentration and is little affected by extracellular potassium concentration. Resting and

spike potential are reported to be unchanged when chloride is replaced by nitrate or other abnormal anion, though the negative after potential was prolonged (Etzensperger, 1956). Even at the end-plate membrane, there is evidence that the effect of the acetylcholine released by the end-plate is predominantly to increase sodium conductance rather than to depolarize the membrane itself to any appreciable extent (Nastuk, 1953).

If the consequence of excitation is a sudden inrush of sodium current, the membrane sodium conductance would have to suddenly increase and its overall impedance fall to a very low value, as was shown by Curtis and Cole to occur in squid nerve. Dubuisson (1937) showed that upon stimulation of frog muscle, there is a sudden transient decrease in impedance, probably associated with the action potential, followed by an increase. Later, the secondary increase was divided into two fractions (Dubuisson, 1950). Katz (1942) observed a decrease in resistance at the end-plate region in association with the end-plate potential and action potential. Jenerick (1953) has shown that polarizing currents alter membrane resistance in a manner similar to that observed in squid nerve. The membrane also exhibits the characteristic rectification, with the resistance diminishing at the cathode and increasing at the anode. Curiously, this rectification is completely reversed if phosphate is omitted from the Ringer solution.

In heart muscle fibers, the spike seems to be dependent on the sodium potential even in the presence of high potassium. The falling phase of the action potential is quite different from that of squid nerve or skeletal muscle, for in heart tissue the resistance of the membrane falls to a very low fraction of the diastolic resistance at the crest of the spike, but then very rapidly increases at several different rates throughout the falling phase (Weidmann, 1957; c.f. Hecht, 1957; Brady and Woodbury, 1957).

7. *Conduction Velocity*

According to Cole (1957), from measurements of ionic conductance data, axon diameter, resistivity of the core, membrane capacity, and ionic potentials, it is possible to predict most of the behavior of the squid giant axon including the velocity of conduction. The ionic conductance data is the major missing information needed for similar calculations for muscle; thus, it is impossible to predict what the velocity of conduction should be in muscle. The older local current theories of Blair (1934), Rashevsky (1938), Rushton (1937), and Offner *et al.*

(1940) led to an equation for the velocity of conduction: $v = SL/\alpha$ where S is a "safety factor" depending on the magnitude of the action potential and rheobase, L is an electrotonic length constant, and α is the time constant of the membrane, the latter assumed to be constant. While we know that neither the "safety factor" nor the time constant of the membrane stays constant, qualitatively the velocity of conduction will depend on the magnitude of the action potential and the characteristic length L. While L for muscle has only been measured for purely electrotonic spread (see under excitation), its use is probably legitimate since Hodgkin (1937) showed in nerve that the extrinsic potential (the electrotonic spread of potential of the action current) is identical, except for its efficiency, to the electrotonic potential set up by an applied current.

Neglecting the variations in safety factor and membrane resistance, Katz (1948) showed that in a large volume of tissue or saline, the conduction velocity on the basis of local circuit theory should be proportional to equation (15).

$$V \sim \frac{\sqrt{\text{radius}}}{C_m \sqrt{R_i}} \tag{15}$$

C_m is the membrane capacity and R_i is the specific internal resistance. The calculated right hand terms of equation (15), in relative units for three tissues having widely differing conduction velocities, approximate the measured velocities (Katz, 1948). Katz found an average velocity of 1.6 m. per second in fibers of M. extensor digitorum IV of the frog, confirming the earlier work of Eccles *et al.* (1941), who found in the sartorius muscle at 20°C. a conduction velocity of 1.6 m. per second.

From equation (15), the velocity would be expected to be approximately proportional to the square root of the diameter of a single muscle fiber. With isolated muscle fibers in Ringer solution, Hakansson (1956) found there is an increase in velocity with increasing diameter. Wilska and Varjoranta (1939a) found that conduction velocity slightly increased with stretch (19% for a 50% stretch) in muscle fibers of M. transversus abdominus from *Rana temporaria*. Martin (1954) found no change in conduction velocity in curarized frog sartorius over a range of lengths from 67 to 120% of resting length. From this fact he argued the membrane is "folded." On the basis of Wilska's results, only a 2% change would be expected in the range from 100 to 120% length and there might well be a "folding" of the membrane for the shorter

lengths. On the "active relaxation" theory, this would be expected in whole muscle where the connective tissue prevents the fiber's re-extension. Whichever result is taken, it obviously is not in accord with equation (15) and suggests that comparisons of stretched versus unstretched muscle might be a good way indirectly to get at conductance controls with presently available methods.

If the time constant *cr* of the membrane remains constant with stretch, the membrane potential will fall unless the cell membrane is recharged. Earlier, Ramsey (1947) calculated the fall in membrane potential to be expected in this case if the membrane is not recharged. On the further assumption that lowering the membrane potential always speeded up the metabolism of the cell, the data of Hegnauer *et al.* (1934) on the oxygen consumption and fall in injury potential in various KCl solutions were used to relate stretch and oxygen consumption. This relation was then tested against the increased oxygen consumption with stretch of the muscle observed by Meyerhof *et al.* (1932). Considering everything, the agreement was as good as could be expected. It is evident now, however, that the muscle maintains its resting potential with stretch (Ling and Gerard, 1949c) so that the increased metabolism probably is utilized to keep the membrane charged. In this respect, Harris (1954) found an increased intracellular extrusion of sodium with stretch except from mid-June to September, but no effect on movements of potassium either out or in (Harris, 1953). Values of the *cr* of the membrane at the anode as calculated from strength duration data by the method of Blair (1941) compare favorably with the values measured by Katz (1948). In one set of unpublished data in which the strength duration curves were determined at different lengths on an isolated single muscle fiber, the *cr* was calculated according to the method of Blair (1941). This experiment was done in Dr. Fenn's laboratory in 1939. The results are shown in Table I.

TABLE I

cr OF MEMBRANE AT DIFFERENT LENGTHS

Time	4 : 45	7 : 05	8 : 05	9 : 05	10 : 15	10 : 50
Temperature °C.	15°	16°	16°	15°	15°	15°
Length as per cent of resting length	100	100	153.5	100	38.6	100
Rheobase	106	103	125	113*M*125	100	126
cr in msec.	20.2	20.1	19.2	16.1	21.2	15.4
Diameter in μ	100	100	62.4	100	149	100

The variations in *cr* are probably without significance for two reasons. One is that a small but constant length of the fiber was held in a tube which was the cathode, the remaining part of the fiber being in a dish of Ringer solution where the anode was located. The changing diameter of the fiber in the region of high current density certainly influenced the area of current exit. Second, the experiment was done in June and there was undoubted condensation occurring, which over a period of 6 hours might well have changed the Ringer solution slightly. This is the only experiment in which *cr* has been calculated at different lengths but it does indicate that probably *cr* remains constant with stretch.

It thus appears that every factor in the local circuit theory would tend to act in a direction to diminish the velocity of conduction with stretch. Since, if anything, it appears to be increased, the conductance controls must be very much more labile than in the unstretched muscle. Comparisons of the parameters of muscle that can be measured, with the intracellular electrode in the stretched and unstretched condition might provide indirect measures of these controls more quickly than measurements made in any other fashion, particularly if combined with alterations of temperature, for Wilska and Varjoranta (1939b) found a five fold increase in conduction velocity between 0° and 36°C. (0.5 to 2.7 m./second).

In isolated single muscle fibers, the increase in velocity of conduction of the impulse with increasing diameter of the fiber is at least in the direction of that expected on the basis of local circuit theory. However, the velocity of conduction of individual muscle fibers in a whole muscle tends to be approximately the same irrespective of the diameter of the fiber. Buchthal *et al.* (1955) have shown that in the human brachial biceps muscle the probable range of diameters of individual muscle fibers is at least threefold but that the velocity of conduction of many individual muscle fibers taken at random has a mean of 4.7 m. per second with a maximal variation of 1.3 m. per second at 36.5° C. They believe that this "synchronization" is in part due to the phenomenon of interaction between adjacent active fibers which tends to "synchronize" the velocities of conduction, as Katz and Schmitt (1940) first demonstrated in nerve. It is, in part, due to slowing of the velocity of conduction because of the high resistance offered by adjacent fibers. The small scatter of conduction velocities at any one particular temperature or stretch seen in Wilska's experiments probably also indicates that the

velocity of conduction is less dependent on diameter of the individual fibers in whole muscle, unless, of course, the belly wall muscle fibers have less variation in diameter than have most muscles.

III. MECHANICS OF MUSCLE

A. Resting Muscle

1. *The Length-Tension Diagram*

When a resting isolated muscle fiber of a frog is stretched passively beyond its rest length l_o (defined as that length at which the fiber develops maximal tetanic tension), it develops a tension which is not linear with the stretch but increases more as the stretch increases. In some instances, the increase in tension was exponential with increase in length (Ramsey and Street, 1940) (it generally was, according to Buchthal (1942)), but many such individual curves could be fitted by a variety of functions. It is of great theoretical interest in interpreting the structure of muscle to decide whether most of the resting tension can be ascribed to sarcolemma (Ramsey and Street, 1940) or whether most is due to fibrillar material. For a recent review of the latter view, c.f. Buchthal (1956). Since the chief arguments advanced by the opposing protagonists have been often reviewed, they will not be further repeated here. The differences may well arise from the circumstance that the experiments were done differently. In the one, the incremental changes in length were small and were carried out very slowly (Ramsey and Street, 1940); in the other, they were attained by quickly stretching the fiber to its new length and then measuring the tension after it had settled down to an "equilibrium" value (Casella, 1951). Buchthal (1956) reviews numerous other mechanical experiments on resting muscle but since their interpretation largely concerns structure, they will not be included.

One experiment on resting tension has a very direct bearing on theories of muscle contraction. This is the comparison of a resting length-tension curve of a normal fiber which actively relaxes with the resting length-tension curve of the same fiber after it has been allowed to shorten so far that active relaxation is abolished (the delta state). The two curves are identical within experimental limits (Ramsey and Street, 1940). If one argues that the energy stored in the bulged sarcolemma is responsible for the active relaxation of the normal fiber, then one would be forced to argue that in the sarcolemma of the delta

state fibers, only circularly stretched elements had become broken. As Dr. Fenn expressed it, it might have "busted its gussets," a term well known to an earlier generation. This suggestion seems unlikely to the author in view of the very complex nature of the sarcolemma as demonstrated both by histologic methods (Bairati, 1937) and by electron microscopy (Reed and Rudall, 1948) but at this time it cannot be disproved.

B. Active Muscle

1. *The Twitch*

a. Excitation—Contraction Coupling. The link or links between excitation and contraction are completely unknown. The subject is so speculative that only a few facts that seem to be generally accepted will be presented here, documentation for which may be found in the following reviews and papers: Sandow (1952); Katz (1950); Huxley (1957); Botts (1957); and Hill (1949b).

There seems to be general agreement that, whatever the link is, it is activated by depolarization of the membrane per se and not by current flow. Taylor (1953) believes it is the excitation of the membrane itself that forms the link, rather than depolarization or current flow. All the evidence indicates that whereas the action potential is propagated, contraction is not—it occurs locally only where the membrane has been depolarized, whether by a normal action current or by the application of depolarizing agents. This localization is in accord with the recent claim (Huxley, 1957) that excitation travels inwards to the myofibrils only at the Z membrane in vertebrate striated muscle and only affects the immediately adjacent half I bands.

According to Sandow (1952), in frog sartorius muscle there is a constant interval between the crest of the spike and the beginning of latency relaxation of about 1.2 msec. at any temperature between 6.6° and 25°C. Presumably this interval reflects some physical process occurring in the membrane before any of the myofibrils are activated, because there is no sign of an active state developing until the beginning of latency relaxation. At this time, presumptively, the myofibrils nearest the membrane become activated and the activation proceeds as a wave inwardly directed until all of the myofibrils have been activated and the maximal active state attained. Sandow estimates that at 0°C., the velocity of propagation of this activation wave is of the order of 0.8 cm. per second. It is significant that the velocity of

propagation of this activation wave apparently has a rather large temperature coefficient. This in itself would argue against any diffusion of an activating substance from the surface membrane, quite apart from Hill's theoretical demonstration that diffusion would be too slow a process.

While ordinary diffusion of an activator substance is probably ruled out, it is difficult to avoid the conclusion that some activator substance or disturbance is liberated at the surface, a substance which does not affect the magnitude of the active state but does affect its duration. Many substances prolong the active state, notably foreign anions such as nitrate, and in all instances rather conclusive evidence is available that their effects are exerted at the surface of the cell. Since, as noted earlier, the negative after potential is prolonged by these foreign anions, the active state may be prolonged as long as the membrane is depolarized below some critical level. Since the latency between the stimulus and the beginning of the latency relaxation is shortened by these foreign anions (Kahn and Sandow, 1950), the active state may well be due to some critically maintained depolarization rather than a spike-activation process as Sandow assumes.

One of the tentative hypotheses that Sandow has advanced is that the active state is brought about by the liberation of calcium ions at the surface, which combine with adjacent myofibrils and activate them. Once activated they would dissociate and by a type of exchange diffusion be passed inwards, activating other myofibrils in turn. The overall inward rate of movement might be faster than ordinary diffusion, particularly if electrostatic forces were set up by the intense electrical field of the action potential. The absence of such presumed electrostatic forces may account for the failure to observe any spread of activation where calcium was released intracellularly by electrolytic means (Niedergerke, 1955).

b. Latency Relaxation. If a frog sartorius muscle is suitably stimulated under a tension of the order of 8 g. and the tension recorder is sufficiently sensitive, about 1.0–1.5 msec. after the stimulus there is a slight fall in tension; the fall is sigmoid in shape and lasts about 2 msec., followed by an abrupt increase in tension. This slight lengthening first observed by Rauh (1922) has been extensively investigated by Sandow (1950). Under normal conditions, this slight lengthening which Sandow calls latency relaxation corresponds to a tension change in a sartorius

muscle of about 20 mg. and a lengthening of the order of a 0.1μ. The total latent period and the extent of latency relaxation both vary in response to a wide variety of circumstances, such as pH, temperature (including temperature inactivation), abnormal anions, previous activity, excess potassium, etc., in a manner which Sandow believes compatible with the assumptions that during latency relaxation, ATP is bound to myosin and probably also hydrolysis of ATP begins to occur (the latency relaxation presumptively being the mechanical sign of the formation of enzyme-substrate complex). The fact that during latency relaxation there is apparently a rapid evolution of heat (Hill, 1949a) accompanied by a transient increase in transparency (Hill, D. K., 1949) is presented as further support of the hypothesis.

Abbott and Ritchie (1951) found that both the magnitude and the duration of the latency relaxation depended on the length of the muscle, being greater the longer the initial length and being absent at lengths about 10% less than body length. While the latter is to be expected, the former is not if the latency relaxation is related to the amount of enzyme substrate formed and hence to the ultimate amount of splitting. This is so because with extension, twitch tension markedly diminishes and maintenance heat diminishes also (Fenn and Latchford, 1933). While the "active state" to be discussed next is well advanced in the period of latency relaxation, showing that the contractile system has already been profoundly modified, it has been suggested that perhaps the latency relaxation itself is a property of parallel elastic elements responsible for the resting tension (Hill, 1951a). Augmentation of latency relaxation by abnormal anions or potassium occurs evidently as soon as they reach the membrane, so that Hill's suggestion, as in nearly all "parallel" arguments, cannot be dismissed lightly. On Sandow's hypothesis, whether magnitude, duration, or area under the curve of latency relaxation is taken as a measure of the magnitude of combination of myosin with substrate, the twitch tension should be in proportion to the amount of enzyme complex formed. Both the magnitude and area under the curve of latency relaxation diminish with lowered temperature, but twitch tension increases.

To the author, the argument that latency relaxation reflects the combination of myosin and substrate complex is inherently weak. The argument that it reflects some profound changes in the properties of the contractile machinery is on an infinitely sounder basis even though Hill's speculation cannot be excluded altogether.

c. The Active State. The onset and time course of the "active state" have been determined in a number of ways. Using as a criterion the maximum load a muscle can support, without further lengthening, after a small quick stretch, Hill (1949b, 1950a) mapped out the onset and time to maximal development of the "active state" in both frog and tortoise muscle at 0°C. Because the velocity of shortening of tortoise muscle is so much slower than frog muscle, the results were much more accurately related to the latent period. It seemed probable (Hill) that the onset of the "active state" coincided with the beginning of latency relaxation, if latency relaxation in tortoise muscle begins at the same phase of the latent period as it does in frog muscle. At all events, decreased extensibility could be detected when about 50% of the latent period was over and the "active state" developed to full activity within a few multiples of the latent period. This, according to Hill, is only a few hundredths of the time required for the isometric twitch to reach the maximum. A little earlier, Hill (1950b) had shown that with tortoise muscle the heat production begins when approximately 50% of the latent period is over. In the frog, sartorius muscle activation heat begins very early also (Hill, 1949a). As he remarks, it is probably a fair inference that latency relaxation, altered extensibility, and heat production all begin simultaneously.

Several other methods have been used to determine the duration of the plateau of the "active state." The minimum rate of stimulation in a maintained tetanus in which no tension changes can be detected has been used as one measure (Mauriello and Sandow, 1953; Ritchie, 1954). Also, the time from the last shock of a tetanus to the time the tension starts to decline (Ritchie, 1954) has been so used. MacPherson and Wilkie (1954) compared the curve of isometric tension rise in a twitch with the curve of a tetanus or a double twitch and took as a measure of the end of the plateau of the "active state" that time at which the curves separated. They gained a little greater sensitivity by differentiating each curve and comparing the differential form. The duration of the "active state" diminished with increasing temperature and to some extent depended on stimulus strength, particularly at low temperatures (c.f. Hill, 1951b). Ritchie (1954) similarly found that the duration decreased with increasing temperature, with a $Q_{10} = 2.2$. Hydrostatic pressure also prolongs the "active state" (Brown, 1936).

A wide variety of agents and treatments have been discovered which affect twitch tension but not tetanic tension. In all of these instances,

presumably the active state has been prolonged or diminished. Hill and MacPherson (1954) showed that the active state is prolonged in muscles which had been immersed in abnormal anions such as nitrate, just as it had been earlier shown that nitrate potentiates the twitch and the depth of latency relaxation (Kahn and Sandow, 1950). Similarly, excess potassium potentiates the twitch and prolongs the active state (Sandow and Kahn, 1952; Mauriello and Sandow, 1953) as does quinine (Lammers and Ritchie, 1955).[1]

The active state may be a function of the absolute refractory period. From some experiments reported by Ramsey and Street (1941b) but unpublished in any public journal, the refractory period of isolated single muscle fibers was determined at different temperatures. The logarithm of the absolute refractory period (A.R.P.) plotted against the temperature in °C. gave a reasonably good straight line though at any temperature, there was considerable scatter. The best straight line was determined by least squares and from it equation (16) was calculated.

$$\text{A.R.P. in msec.} = 25.12 \exp. (-0.09T) \qquad (16)$$

T = temperature in °C. Both the general shape of the curve and the magnitude of increase of the A.R.P. with lowered temperature are similar to that found by Bazett (1908) on whole sartorius muscle.

The duration of the active state at three different temperatures at a stimulus strength five times threshold was interpolated from Fig. 4 of MacPherson and Wilkie's (1954) paper and compared to the A.R.P. as calculated from equation (16). The results are shown in Table II.

From the facts, that the active state is approximately twice as long as the A.R.P. and that they both have the same Q_{10} (2.48 for A.R.P., 2.46 for average Q_{10} for active state (A.S.) as calculated from the above figures), it may be suggested that the duration of the A.R.P. is governing

[1] Falk and McGrath (1958), report that if ferrocyanide replaces chloride in Ringer solution, the response to a single stimulus reaches tetanic tension and the twitch may last some 1.5–5 sec. Other anions also prolong the active state, the order of effectiveness being ferrocyanide ~ sulfate ~ acetate ~ iodide. Some fibers show only a single action potential but many fibers show repetitive electrical activity which is a consequence of the increased negative after potential. The mechanical response outlasts the period of repetitive activity by several seconds. A critical concentration of chloride practically abolishes the effect. Chloride concentration apparently is related to the duration of the active state both in the presence and absence of other ions. They suggest that perhaps the active state is normally terminated by chloride entry during the after-potential but that other ions may substitute in the order of their relative membrane permeability.

TABLE II

ABSOLUTE REFRACTORY PERIOD AND ACTIVE STATE

Temperature, °C.	0°	9.5	20.2
A. R. P. msec.	25.12	10.6	4.0
Duration A. S., msec.	56.0	19.0	10.0
A. S./A. R. P.	2.23	1.8	2.5

the duration of the active state. Enhancement of twitch tension occurring as the consequence of previous activity is always accompanied by an increase in A.R.P. (Ramsey and Street, 1941b; Ramsey *et al.*, a); but in two successive runs on the same fiber, the increase in A.R.P. for a given degree of potentiation of tension is smaller the first time than it is for a later run, indicating a system of considerable lability. This lability, however, may be a peculiarity of the isolated muscle fiber due to the favorable diffusion conditions and the fact that it is in Ringer solution instead of plasma. This situation might easily lead to the slight loss of some essential constituent of the excitation—contraction coupling system so that a longer A.R.P. was required to maintain the active state.

It is an unfortunate fact that we cannot use the records of the single muscle fiber data to correlate the duration of the A.R.P. with the duration of the "active state" in the manner MacPherson and Wilkie (1954) did. We have both the twitch and double twitch records and the threshold shock strengths required for the entire relative refractory period. It is clearly evident from these records that the interval between the first and second shock has a profound influence on the rate of rise of tension of the double twitch. This influence is less the lower the temperature, so that MacPherson and Wilkie (1954) are probably justified in using their method of estimating the end of the plateau of the "active state," since they worked at 0°C. Most of our records are not at 0°C. At 13.5°C., for example, in one experiment where the A.R.P. was 7.0 msec. as judged by noting when a second shock caused a small increment of tension over twitch tension, the single and double twitch had the same slope. Applying the criterion of separation of twitch and double twitch, this would signify the end of the plateau of the "active state." If, however, the second shock is applied a few (2–3) msec. later, the slope of the double twitch is very much steeper than the slope of the single twitch. If the second shock is applied at a later interval, the slope of the double twitch gradually decreases, until it

equals the twitch slope when the second shock is applied 37 msec. after the first. Under these circumstances, it is impossible to use this method for estimation of the duration of the "active state."

2. *Tetanus*

a. Maximum Isometric Tension. If a single striated frog muscle fiber is stimulated tetanically at the length where maximum tension is developed, it will develop a tension of approximately 3.5 kg. per square centimeter, if in the calculations the average cross-sectional area of the fiber is used (Ramsey and Street, 1940). At best, this can only be an approximate value because the cross-sections of most fibers are so irregular, but it agrees well with other data on single muscle fibers, e.g. 3.0 kg. per square centimeter (Casella, 1951) and is similar to values observed in whole muscle.

This maximum developed tension is virtually invariant. In whole muscle, a wide variety of procedures and agents that markedly affect twitch tension have no effect on this maximum tension. It is practically invariant with temperature; 9% per 10°C. (Casella, 1951). Washington *et al.* (1955) felt that probably most of the small variation observed by them lay within the limits of experimental error. There are a number of reports in the literature in which it is claimed that there is a marked influence of temperature on the maximum tetanic tension developed by whole sartorius muscle. In most instances, these results probably arose from faulty stimulation, either by failing to arrange conditions to insure that muscle was being stimulated directly or by using too high a frequency of stimulation at the lower temperature. When muscle fibers at any temperature are stimulated at too high a frequency relative to that required for a smooth tetanus, the tetanus rapidly "cuts off," the effect being most marked at the lower temperatures (Ramsey and Street, 1941a). In an assemblage of fibers, this effect would strongly influence the results if the same frequency of stimulation were used at all temperatures. Washington *et al.* (1955) discussed this effect, but neglected to mention the fact that the frequency of the Harvard inductorium used was adjusted by loading the armature to compensate for it. In contrast to the invariant quality of the magnitude of the tension developed (always assuming the frequency of stimulation was adequate), the rate of rise of tension to the plateau and the rate of relaxation have considerable lability.

The rate of relaxation from an isometric tetanus is extremely labile,

being markedly prolonged by low temperature, previous activity, and by agents which increase twitch tension in general. The temperature coefficient of the rate of relaxation is approximately 3 to 4, paralleling to a close degree the maintenance heat (Ramsey, 1944). It should be noted that, following the usual terminology, the rate of relaxation of an isometric tetanus refers to the rate at which tension is lost following the last shock of an isometric tetanus. It does not refer to cessation of activity of an isolated fiber, for when the tension is zero, the fiber is still actively relaxing (Ramsey, 1944). The tension becomes zero the instant any part of the fiber ceases contracting, but active lengthening of a shortened fiber lasts 2 to 3 times longer than the time required for all tension to disappear.

b. The Series Elastic Component. It is quite generally held that contracting muscle behaves as a two component system consisting of some type of contracting machinery in series with an elastic element. Hill (1950c) estimates that the total stretch of the series elastic element of a sartorius muscle is equal to 0.04 L_0. The relative inextensibility of tendon makes it unlikely that much of the series elasticity could be ascribed to stretch of the tendon. A further indication that it is not due to tendon is the fact that when a muscle fiber develops tension at a length that is shorter than rest length the rate of development of tension is progressively and markedly slowed, the greater the initial shortening permitted (Ramsey and Street, 1940). This suggests that additional compliance was being added linearly with the extent of shortening permitted. While there may be other explanations, if this latter one is true, one is compelled to assume that the series elastic element in some manner resides in the contractile machinery itself.

One possible mechanism by which the series elastic element could arise and increase with shortening has been suggested (Ramsey, 1955). This mechanism supposes that normal muscle behaves as a high polymer that, statistically speaking, is like a methyl vinyl ketone type having two reactive groups on each substituent. As Flory (1942) and Wall (1942) demonstrated, when pairs of neighboring substituents of a long chain polymer of this type condense intramolecularly with one another, irreversibly and at random, $1/2e$ (where e is the exponential $= 2.718$) or 18.4% of substituents fail to react because they become isolated. In the random reaction, ultimately there would be some pairs of carbonyls facing each other, and hence these could not enter into the conden-

sation reactions. This same degree of isolation will also occur in the condensation of true copolymers if only one of them bears neighboring reactive substituents in the 1–2 or 1–3 positions in the hydrocarbon chain (Wall, 1942). In Ramsey's analysis, it was assumed that in free shortening, the reaction between contiguous reactive groups was random and therefore the reaction of tail reactive groups of a monomer with the head reactive group of its dimer (contiguous monomer) is as probable as the reaction of any pair within a monomer. It was assumed that the isolation of groups increased with shortening, becoming maximal and equal to 18.4% when a muscle fiber had shortened to 18.4% of its rest length, and that these isolated groups gave rise to the series elasticity of muscle.

c. The Isometric Length-Tension Diagram of Tetanically Stimulated Single Muscle Fibers. The isometric length-tension developed diagram of single muscle fibers is astonishingly reversible and constant over a range of lengths from 2/3 L_o to 2 L_o, provided care is taken to stimulate the fiber in such a manner as to ensure that the fiber shortens uniformly along its length when it is allowed to shorten and then develop tension. When the fiber is allowed to shorten unequally by stimulating it at one end, the initial tension developed is always higher than is the case for the uniformly shortened fiber. If stimulation of an unequally shortened fiber is continued, shortening along its length is gradually equalized and simultaneously the tension falls to the value for the evenly shortened fiber. This behavior is perfectly consistent with an invariant length tension diagram since the force developed by a single muscle fiber *per unit cross-sectional area* reaches a maximum at 1.20 L_o (Ramsey, 1947) and the lengthened end would pull out the shortened end. However, it is somewhat surprising that it takes an appreciable time to do this. The above behavior was used in an earlier analysis (Ramsey, 1944) to emphasize that a stimulated muscle at a given length may have two totally different configurations of its molecules. Considered as a potential energy machine, two different amounts of energy would have been liberated. It was pointed out that if the load governed evenness of shortening on molecular dimensions, this might explain the Fenn effect. It might also account for the different length-tension diagrams (isotonic, isometric, etc.) obtained by Buchthal's group (Buchthal, 1956). While uneven shortening occurs in whole muscle also (Fischer, 1926; Sandow, 1936), the effect is smaller because of the restrictions

offered by the connective tissue. When precautions are taken to stimulate at a number of points along a whole muscle, the isotonic and isometric length-tension diagrams coincide for lengths below body length (rest length) (Abbott and Wilkie, 1953). Isotonic data on whole muscle (data of Fenn) agree reasonably well with the isometric length-tension diagram of single muscle fibers in the range 2/3 L_o to L_o, if it is assumed that the connective tissue offers resistance to shortening, preventing it from further shortening at a length equal to 2/3 L_o

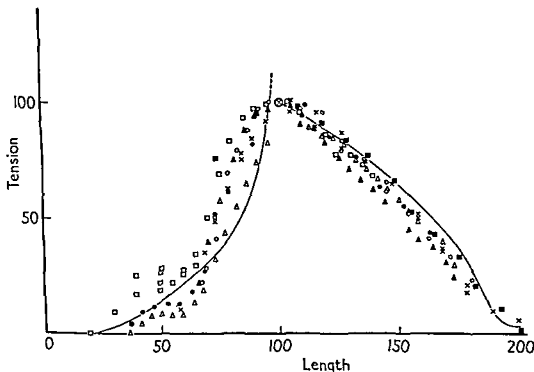


FIG. 8. Length-tension developed diagram. Ordinates: tension developed (total minus resting) in percentage of maximum developed. Abscissa: length in percentage of resting length. Solid lines: theoretical diagram. Symbols: data of Ramsey and Street. From Ramsey (1955), p. 699, Fig. 2.

(Ramsey, 1944). Similarly, the quick release experiments on single muscle fibers quoted in the same article give the same length-tension diagram. While not investigated over its entire reversible range, the isometric length-tension diagram is invariant with respect to shape and magnitude (within the limits of experimental error) with temperature (Fig. 8). In sum, the author cannot agree with Buchthal that the relation between length and tension is ambiguous.

In the region of lengths greater than rest length, there is no similar

certainty that a muscle shortening isotonically from an extended length with preload will shorten to the length characteristic of that tension on the shortened side. In single fibers, there is some evidence that it will not do so (Buchthal and Kaiser, 1951). It is of the greatest theoretical importance for our understanding of the muscle machine to establish this point, but the attainment of unambiguous results presents formidable technical difficulties either at the single fiber or whole muscle level.

d. The Delta State. If a single muscle fiber is stimulated and allowed to shorten and then develop tension at the shorter length, when stimulation ceases it very actively relaxes, provided it was not allowed to shorten more than 1/3 of its rest length. If it is allowed to shorten somewhat more, it fails to relax at all. After further stimulation, it will shorten to 19% L_o but does not develop any tension (within the sensitivity of the lever of approximately 1 mg.) except for one experiment, at lengths below 36–40% L_o. Even at 19% L_o, although no tension was registered by the lever, one could visibly see the fiber was active upon stimulation, shortening at one end and stretching at the other and *vice versa* continually during the tetanic stimulation.

Upon re-extension (except for one experiment), no tension was developed upon stimulation until a length of 36–40% was reached. At greater lengths, more tension was developed (reaching a maximum at L_o, which, however, was only 1/2 the maximum of the normal fiber. At lengths greater than rest length, the tension developed decreased and generally was zero at 1.8 L_o. The best re-extension curves approximated an hyperbola. An extended fiber does not repeat this curve when stimulated and allowed to develop tension at successively shorter lengths; instead, a large and variable "hysteresis" was observed. At all lengths, the fiber developed tension very much more slowly, and at most lengths developed less tension than at corresponding lengths on the re-extension curve. Even on the re-extension curve, the rate of development of tension is very much slower than that of the normal fiber at any length (Ramsey and Street, 1940). It requires little force (less than 1 mg.) to re-extend a delta state fiber from its most shortened length to L_o. Above L_o, the resting tension curve is the same as that of the normal fiber.

When a normal fiber is allowed to shorten below 2/3 L_o, the striations remain perfectly regular in so far as it could be determined with the optical system we used. Upon re-extension, irregular bands of close

striations (contracture bands) interspersed with normal bands of striations appeared. When re-extended to L_o, all the contracture bands disappeared, leaving what appeared to be grossly "normal" striations. High power examination however, showed that these "normal" striations had a "fuzzy" appearance due to a slight misalignment at the Z membrane of each Cohnheim bundle. Irrespective of length, whenever a delta state fiber was allowed to shorten, contracture bands would form. These, however, were not constant but would form at one point and then would be pulled out and would reform at another point continuously during stimulation. When stimulation ceased, the last formed contracture bands persisted without further change. Not all fibers will shorten far enough to go into the delta state. This is particularly true of fibers having a tough sarcolemma, such as fibers from the semi-tendonosus of American toads; it also happens occasionally with frog fibers.

The finding of Huxley and Hanson (c.f. this volume) that the Z membrane approximates the A disc (the I or actomyosin filaments being completely drawn into the A disc) at 65% L_o provides a probable morphological basis for the delta state. Their finding also that the Z membrane may be the means by which excitation is conveyed to the contractile filaments might also have a bearing on the delta state, but this is more problematical. For one thing, while the alignment of Cohnheim bundles at the Z membrane is not perfect in the delta state fiber, it is possible in some normal fibers that are considerably stretched to $>$ 160% to observe a similar misalignment, particularly if there is some inequality of the sarcolemma along its length. These fibers develop somewhat less tension upon return to L_o than they did before stretch but they will repeat the new value after re-extension and return. In no instance do they resemble fibers in the delta state with respect to physiological properties.

Apparently, the misalignment of the Cohnheim bundles in the delta state fiber has no influence on the strength-duration curve or on the time constant of the membrane (c.f. Table I). Taking all of the evidence into account, it does not seem likely that the misalignment of the Cohnheim bundles, and hence some disorganization of the Z membrane, can alone be responsible for the delta state, although this is not ruled out. If excitation is conveyed inward via the Z membrane, this disorganization might very well explain why when a delta state fiber shortens it always forms contracture bands which are continually

changing. It might well be that regions of the fiber where the Z membranes are less disorganized contract first and that the more disorganized regions contract later, pulling the first formed contracture bands out and in turn forming contracture bands themselves.

The delta state in the usual reversible range of shortening may make use of a mechanism of shortening such as the Huxleys propose, but be disorganized by the disorganization of the Z membrane. However, at lengths less than 2/3 L_o, there must be some other mechanism of shortening involving a more fundamental change. The delta state fiber shortens to 19% L_o. It was suggested that if muscle behaved like high polymers of the type previously discussed, perhaps this shortened length represented the reactive groups isolated or 18.4% L_o (Ramsey, 1955). In this respect, it is noteworthy that a delta state fiber will not develop tension at lengths greater than 1.8 L_o. One might suppose that in the delta state fiber, these isolated reactive groups are permanently isolated. Acting as a large compliance, this would account for the slowness of development of tension in the delta state. Similarly, it would account for the fact that no tension is developed at lengths less than 36% L_o for by the hypothesis presented, $L_o/2e$ (e = exponential) units equal to 18.4% L_o in length would be contracting against a series elastic element also $L_o/2e$ long. This series elastic element, unfolding to twice this length when stretched by the contracting units, would create a continuous see-saw of folding and unfolding units. It is interesting that Huxley and Hanson (1954) found that the dense zone formed in the middle of the sarcomere during shortening splits at about 65% L_o and approaches the incoming contraction bands, merging with the latter at about 30% L_o.

Discussion of the force velocity relationship of muscle will be taken up in the section devoted to heat of muscle.

e. Active Relaxation. In order to conveniently record active relaxation of a normal isolated muscle fiber and to demonstrate its absence after the fiber had been converted to the delta state and re-extended to rest length, it was necessary to use short stiff muscle fibers (Ramsey and Street, 1941a). With longer fibers, the active relaxation of a normal fiber is very dramatic, the fiber whipping the Ringer solution in the fashion described by Fenn (1945).

The important question to answer is whether the active relaxation is a fundamental property of the muscle machine or a consequence of

some occurrence concomitant with shortening. The identity of the resting tension curves of the same fiber in both normal and delta states argues against it being a restoring force due to a bulged sarcolemma. Different fibers have widely different resting-tension curves, indicating different sarcolemmal strengths, but all exhibit active relaxation and as judged by eye there was no difference in speed of relaxation among them. The rate of relaxation from an isometric tetanus is greatly slowed by lowered temperature; the Q_{10} is between 3 or 4 and the rate of active relaxation, though not measured, presumably also has a similarly high Q_{10}. If sarcolemmal forces were causing the active relaxation, one would have to assume that the viscosity increase with lowered temperature had the same temperature coefficient, because there is ample evidence that the effect of temperature (0–30°C.) on connective tissue is slight. The prolongation of relaxation as a consequence of previous activity would similarly be difficult to ascribe to hysteresis effects in the sarcolemma, because the rate of development of tension in an enhanced twitch is if anything faster than in a unenhanced twitch.

It has been argued that the imbibition of water brought about by the increased osmotic pressure occurring in activity might provide the thrust in relaxation, if some strictly circular elements in the sarcolemma were bonded by internal "cross-ties" (Hill, 1949c). However, if this were the case, the rate of relaxation following a tetanus should be faster than following a twitch, whereas in fact it is far slower. Hill (1949c) has measured the latent periods of whole sartorius muscles at different lengths and found them to be the same at shorter lengths as they were at body length. From this fact, he argued that if active relaxation occurred, the fibers in the muscles at shorter length would be kinked and the slack would have to be taken out of them before tension could be registered by the lever and hence the latent period should be longer in the shorter muscles. In the author's view, the complexity of the connective tissue is so great that the situation is analogous to a brush pile—no matter how small the twig you attempt to withdraw from the pile, a disturbance is created which is transmitted to the whole of it instantly. Similarly in muscle, contraction of any part would be communicated to a sensitive lever system by the complex connective tissue network. According to Abbott and Wilkie (1953), the maximum velocity of shortening is less if the muscle is at a length less than body length. One would expect, therefore, that the latent period of the shortened muscle would be longer than the latent

period measured at rest length. The fact that the latent period is constant argues that the fibers in the muscle at rest at lengths less than body length are in reality probably not shorter than rest length.

In summary, there is a very vigorous active lengthening of a single muscle fiber following cessation of stimulation. While the author believes that the evidence favors the view that this active lengthening probably is due to an active process of the contractile machinery, he would be the first to admit that the evidence is not unequivocal.

IV. HEAT

Throughout the 1920's and the 1930's, muscle was conceived of as a machine which at rest had a fixed store of potential energy which when stimulated could be liberated and degraded as heat or allowed to do work. The observed mechanical behavior during contraction was explained on the basis of presumed "visco-elastic" effects. The chief model assumed that muscle could be represented as an undamped elastic element in series with the contractile machinery, which could be represented as a spring damped by viscosity of the medium. While this view of muscle, pushed to extremes by the Hill school, was widely prevalent, there were some noted dissenters of whom probably the most notable was W. O. Fenn. Fenn (1924), working in Hill's laboratory, showed that when a muscle shortened and did work, it gave out more total energy than if shortening were prevented. For a given degree of shortening, the extra energy liberated was approximately equal to the work done. He further showed that for a given load the extra heat liberated increased with shortening. This Fenn effect (as it is called by everybody except Fenn) was the most probable explanation of the "viscous" effects. Fenn stated that these phenomena had little to do with viscosity per se, but merely reflected the speed with which chemical energy could be mobilized to do different amounts of work. While Fenn's techniques must be considered crude when compared with methods and instruments developed later by Dr. Hill and Mr. Downing, it is true that his results contain to a remarkable degree the hard core of the heat story.

The "viscous-elastic" hypothesis probably was carried to its ultimate conclusion when Hill (1927) calculated that the maximum velocity of running was limited to that speed at which virtually all of the work of the muscles was used in overcoming their own viscosity. Shortly thereafter, Fenn (1930) showed that if in a runner one calculated the work

of accelerating and decelerating the limbs, the rise and fall of the center of gravity, reaction of the ground, etc., this total work substracted from that estimated by indirect calorimetry left little to be used for overcoming viscosity. In an after loaded isotonic contraction, any presumed undamped series element would remain constant during shortening and the actual shortening characteristics should be due to the contractile element alone. Fenn and Marsh (1935) investigated the force-

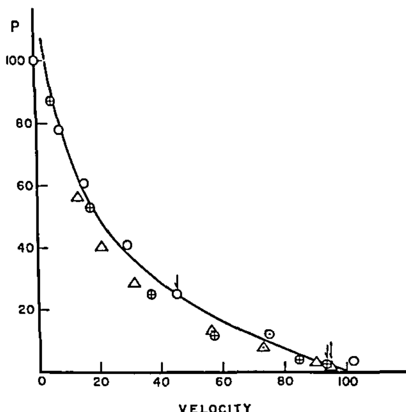


FIG. 9. Solid line: theoretical force-velocity relation. Ordinates: force in percentage of maximum developed at rest length. Abscissa: velocity in percentage of theoretical maximum. Crossed circles: averaged data of Fenn and Marsh (1935) (sartorius 0°C., Table I and Fig. 4). Open circles: data of Hill (1938) (p. 177; on sartorius 0°C.). Open triangles: data of Katz (1939b) (p. 48 on sartorius 0°C., graph a, Fig. 1). Data redu... one particular load. ... was made to fit the t...

velocity relationship under these conditions and found that the decrease of force with increased velocity of shortening was not linear as it should have been on the simple viscosity basis but instead decreased more or less exponenitally (Fig. 9). [While these results rule out Newtonian (simple) viscosity, they are in accordance with non-Newtonian viscosity.]

A. Initial Heat

In a long series of papers, all published in the same journal and beginning with the classic paper of 1938, Hill reinvestigated the heat liberated by muscle under a wide variety of conditions. Though not individually cited, it will be understood that all results quoted derive from Hill's papers unless otherwise acknowledged.

By definition, the initial heat is the total heat liberated during the contraction phase of a muscle contraction. During relaxation, no heat is liberated by the muscle unless work is done on the muscle. The energy stored in a load in isotonic shortening will reappear as heat in relaxation if the load re-extends the muscle. Similarly, the energy stored in the stretched series elastic component reappears as heat during relaxation. The initial heat is the same under aerobic and anaerobic conditions.

1. *Isometric Conditions*

When a muscle is stimulated, there is an initial rapid liberation of heat, beginning probably at the time of latency relaxation and well before any tension is developed. This moiety of heat, which Hill calls "activation" heat in the belief it is concerned with the establishment of the active state, is repeated in a tetanus with each stimulus. In a maintained tetanus, it is identical with the older term "maintenance heat." Accompanying the activation heat, there is a slower evolution of heat beginning at the time tension starts to develop; this is due to "shortening" heat liberated as the shortening contractile units stretch the series elastic element and develop tension. Upon relaxation, the energy stored in this stretched series elastic element appears as heat.

2. *Isotonic Contractions*

Hill found that when a muscle shortened, it liberated a constant amount of heat per centimeter of shortening and that this amount was independent of the extent of shortening, of load and hence of velocity of shortening, of temperature, of the time during a tetanus a muscle is permitted to shorten, of the work done, and according to Brown (1941) of the hydrostatic pressure and pH. It thus seems to be a fundamental muscle constant which in Hill's terminology $= a$, or for comparison with other muscles of different cross-sectional area, a/P_0 where P_0 equals the maximum isometric tetanic tension at rest length. Figure 10 illustrates constancy of shortening heat under a few of the above conditions

and also illustrates that the activation heat is the same for isometric or isotonic contraction. Since this latter is true, it is legitimate to subtract the activation heat from the total initial heat and consider the result the total extra energy mobilized over and above isometric when a muscle shortens and does work. This is the procedure Hill used in deriving his empirical but justly famous characteristic equation.

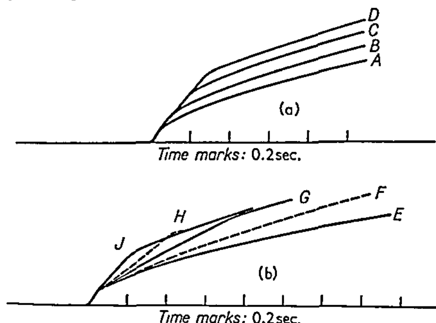


FIG. 10. Heat production during isotoned shortening from the start. Curves traced from galvometer records and superimposed. Tetanus at 0°C.: muscle 32½ mm. long, 85 mg.: time, 0.2 sec. (a) upper: Shortening different distances under constant load of 1.9 g.: A, isometric; B, 3.4 mm.; C, 6.5 mm.; D, 9.6 mm. (b) Lower: Shortening constant distance 6.5 mm.; under different loads: E, isometric; F, 31.9 g.; G, 23.7 g.; H, 12.8 g.; J, 1.9 g. From Hill (1938), p. 158, Fig. 6.

3. *The Characteristic Equation*

If a is the shortening heat per centimeter and x is the distance shortened in centimeters, then the shortening heat will be equal to ax. The work done in lifting the load P will be Px. The total excess energy liberated will then be expressed as equation (17).

$$(P+a)x \tag{17}$$

Experimentally, Hill found the rate of extra energy liberation was a linear function of the load, increasing as P diminishes and being zero when the load $P = P_o$ therefore

$$(P+a)\frac{dx}{dt} = b\,(P_o - P) \tag{18}$$

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Hill found that when a muscle shortened, it liberated a constant amount of heat per centimeter of shortening and that this amount was independent of the extent of shortening, of load and hence of velocity of shortening, of temperature, of the time during a tetanus a muscle is permitted to shorten, of the work done, and according to Brown (1941) of the hydrostatic pressure and pH. It thus seems to be a fundamental muscle constant which in Hill's terminology $= a$, or for comparison with other muscles of different cross-sectional area, a/P_0 where P_0 equals the maximum isometric tetanic tension at rest length. Figure 10 illustrates constancy of shortening heat under a few of the above conditions

bility referred to is solely in reference to the degradation of the work into heat—the muscle is unharmed.

Fenn (1924) showed that when a muscle was stretched during the contraction phase, less total energy was liberated, and he concluded that when a muscle does negative work the excess energy liberated was negative. This has been further studied by Hill (1938), Abbott *et al.* (1951) and Abbott and Aubert (1951). If a contracting muscle is stretched by forces up to or less than about 1.5 times maximum isometric tension, the muscle slowly lowers the load (i.e. lengthens) and the heat production is less than isometric heat production. The work done on the muscle does not appear as heat and the actual heat production is less. There is a negative heat of lengthening corresponding to the heat of shortening (though the constant of proportionality *a* thermal is up to 6 times as great in the former as in the latter case (Wilkie, 1954). The confirmation of Fenn's earlier work plus the finding that the shortening heat per centimeter was the same for all velocities of shortening effectively disposed of the "viscous" elastic model or at least relegated it to a very minor rôle, as Fenn had long maintained.

5. *Recovery Heat*

In oxygen, the recovery heat is rather closely equal to the total energy production during contraction (initial heat and work done by the muscle) under a wide variety of conditions. Under anaerobic conditions, there is first an absorption of heat followed by an evolution of heat, the total evolved amounting to approximately one-twentieth the recovery heat when the muscle is in oxygen (Hill, D. K., 1940a, b). The oxidative and anaerobic recovery heats are both reduced if the pH of the Ringer solution is 6.0, when presumably the formation of pyruvate or lactic acid is prevented. Whether at a normal pH of 7.2 or at a pH of 6.0, the oxygen consumption closely parallels the oxidative recovery heat (Hill, D. K., 1940b).

V. A GENERAL ANALYSIS OF CONTRACTION

Our fundamental knowledge of the muscle machine is obviously still so imperfect (c.f. Morales *et al.*, 1955) that descriptions that encompass much of the energetic data of muscle within a relatively small compass still have utility in suggesting experiments on living muscle. Earlier, the author (Ramsey, 1944) showed that the energetic data which supported the Fenn-Hill hypothesis that energy for contraction was

where b is the proportionality factor which has the significance of an absolute rate of energy liberation. The constant b has the dimensions centimeter per second, hence depends on muscle length. The b of a muscle L times longer than one 1 centimeter long will be bL. Hill (1938, p. 161) quotes a mean value of b/L of 0.33 per second for a frog sartorius at 0°C. It increases about 2.05 times for a rise of 10°C. Both a and b remain constant at all lengths (Abbott and Wilkie, 1953). Since $dx/dt = v$, the velocity of shortening equation (18) may be rearranged to give equation (19).

$$(P+a)(v+b)=(P_o+a)b=\text{constant} \tag{19}$$

However interpreted, the beauty of this characteristic equation of Hill's is that the two constants can be evaluated independently, either from heat measurements alone or from the mechanical force-velocity relationship, since equation (19) states the relation between force and velocity. The actual data of the force-velocity relationship are fitted equally by a number of functions but the only justification for abandoning Hill's empirical relationship is to establish a more fundamental basis that could account for the facts as well.

4. *Heat of Lengthening*

The effects on heat production of stretching a muscle are complicated. Maintained stretch of a resting muscle increases the resting metabolism (Meyerhof *et al.*, 1932) and heat production (Feng, 1932). In a certain range of small extensions a small quick stretch results in a quick burst of heat, or in a quick release from a stretched position, an absorption of heat (Feng, 1932). This is what would be expected if resting muscle had rubberlike elasticity but the actual situation is much more complicated, for Hill (1952) showed that in such quick stretches (and releases) the heat given off (or absorbed) is much greater than the work done on (or done by) the muscle and that the evolution (or absorption) of heat lagged considerably behind the stretch or release.

In contracting muscle, the clearest quantitative result is obtained when a shortened muscle is re-extended by a load during the *relaxation phase*. The work done by the load in re-extending the muscle appears quantitatively as heat. Similarly, if contracting muscle is re-extended in any phase of contraction by too great a load, the muscle "gives" or "irreversibly" lengthens Hill (1938) and under these circumstances the mechanical work is degraded quantitatively into heat. The irreversi-

reaction in the polymer are both markedly increased by an increase in temperature. (e) The hypothesis accounts for the series elastic component of both qualitatively and essentially quantitatively[3] and predicts that the extensibility should increase with shortening. (f) The hypothesis also accounts for the fact that a fiber can shorten to 19% L_o but below 36–40% L_o develops little or no tension. (The shortest length observed in ryanodine contractures in numerous experiments was 13.5% L_o [unpublished]. If this same argument were applied to contractures of this kind, one would assume that the muscle was behaving like a long chain polymer having reactive substituents on neighboring 1–2 positions, for Flory showed that in intramolecular condensations of this type $1/e^2$ or 13.53% of the substituents fail to react because they become isolated.)

A. The Normal Length-Tension Diagram and the Force Velocity Relation

Let us consider the ultimate contracting unit as a long thread having reacting substituents evenly spaced along its length. Upon stimulation, these reacting substituents are "unmuzzled" and in free reaction, neighboring pairs react randomly and irreversibly, leading to shortening, isolation of groups, and evolution of shortening heat as their internal energy is dissipated in the reaction. In terms of reaction rates formulated from statistical mechanical considerations, the specific rate of reaction per mole would be given by equation (20).

$$r = \frac{kT}{h} \exp. (-\Delta F/RT) \tag{20}$$

Here k is Boltman's constant, h is Planck's constant, ΔF is the free energy of activation, and R and T have their usual significance. The transmission coefficient is assumed equal to 1.0.

Suppose a mole of contracting units were lined up on one thread, then the velocity of shortening will be given by the net number reacting per second multiplied by the distance S each shortens—equation (21).

$$V^{net} = \frac{kTS}{h} \exp. (-\Delta F/RT) \tag{21}$$

[3] An arithmetic error was made in the calculation of the extensibility from Wilkie's data in the original paper. The corrected extensibility should be 0.025 centimeter./per Megadyne instead of 0.01. Wilkie (1950).

supplied from chemical stores during the contraction phase—relaxation being purely passive—could equally well be used to support a thermodynamic cycle that was exactly the opposite. Later, (Ramsey, 1955) reaction rate principles[1] were applied to this same cycle and in addition it was assumed that shortening muscle behaved in a fashion statistically similar to some infinitely long "head to tail" polymers such as the methyl vinyl ketone type discussed in Section IV, B, 2, b. In polymers of this latter type, the reacting substituents are on the 1–3 position of the hydrocarbon chain and the reaction consists of an intramolecular condensation of a methyl group of one substituent with the carbonyl group of its neighboring substituent, with the elimination of one molecule of water. Since the reaction is random and irreversible, it ultimately leads to isolation of some groups or pairs of carbonyls facing one another, the whole containing $1/2e$ or 18.4% oxygen.

Whatever the mechanism of contraction is in the normal physiological range, it is impossible to avoid the conclusion that in the delta state fiber, which can shorten to 19% L_o, there must be a large degree of folding of the contractile machine. Originally, it was assumed that if it were not for the restriction of the sarcolemma the folding process could continue until each ultimate myosin chain would fold to a globular protein. In the present analysis, essentially the same assumption is made, but in addition it is assumed that it is not the sarcolemma that restricts further shortening but the fact that the reaction is complete and the final length represents chiefly the combined length of those reacting substituents that had become isolated in the random and irreversible reaction.

Some of the considerations that led to the adoption of this point of view are the following: (a) If the shortest length (19% L_o) of a delta state fiber represents the length of the isolated groups then the degree of isolation is the same as in the high polymers discussed (18.4%). (b) The amount of shortening of the muscle and the extent of the reaction of the polymer are little affected by a temperature change of 30°C. (at temperatures compatible with life in the case of muscle and at temperatures compatible with the reaction proceeding at all in the case of the polymer). (c) The energy released as heat is practically proportional to the shortening in the muscle and to the extent of reaction in the polymer. (d) The velocity of shortening of muscle and velocity of

[1] Reaction rate principle were first applied to discussion of an energetic cycle of muscle by Buchthal and Kaiser (1951), Polissar (1952) similarly used them.

in excess of the average by $3p$, therefore the total excess energy (E) mobilized from the total store is shown in equation (24).

$$E = 3pP_oL_o \exp.(-3p) \tag{24}$$

The work done w is expressed as formula (25).

$$w = pP_oL_o \exp.(-3p) \tag{25}$$

The total energy mobilized into the reacting units is three times the work done. Differentiating w with respect to p in equation (25) and solving for a maximum, the condition of maximum work is that p equals 1/3. Substituting this value in equation (24), the maximum energy in the reacting units is equal to P_oL_o/e, one third of this being stored in the load.

One must now determine how much energy was wasted as shortening heat (a). According to Hill (1938), a varies from 0.2 to 0.4 P_o. The exponent in equation (23) may be written as P/a, in which case the shortening heat per centimeter adopted here would be 0.33 P_o, the value shown previously to be the best one that accounted for both the length-tension diagram and force velocity data on the basis of the proposed thermodynamic cycle. This is an average value. It is suggested that if there were no isolation of groups with shortening the true heat of shortening would be 0.368 P_o or e would equal $e = 2.718$. In free shortening 18.4% of the reactive groups become isolated and hence the shortening heat would decrease to 0.3 P_o. Shortening heat would thus be least with free shortening and would increase with increasing load; hence the value of 0.33 P_o assumed here represents an average value.

Thus for a load equal to $p = 1/3$, one third of the energy mobilized in the reacting units is stored in the load, one third is degraded as shortening heat, and one third is left as potential energy in the molecular structure. The amount of potential energy left in the structure for any other load p may be determined by subtracting the work done and the heat of shortening from the total energy mobilized as given by equation (24). This is equal to equation (26).

$$P_oL_o \exp.(-3p)\,[2p - 1/3] \tag{26}$$

By inspection of equation (26), it is readily seen that for all loads from $p = 1/3$ to 1.0, the potential energy left in the structure is equal to or greater than the work done. For all loads less than $p = 1/3$ where

In the presence of a load Δp on the contractile unit, the velocity of shortening would be diminished by the factor exp. $(-S\Delta p/kT)$. Thus equation (21) becomes equation (22) for the initial condition.

$$\max V_{\Delta p} = \frac{kTS}{h} \exp. \left(\frac{-\Delta F}{RT} - \frac{S\Delta p}{kT} \right) \tag{22}$$

Since S, ΔF, Δp, and the mass that constitute a mole of contracting units are unknown, the data of muscle are fitted empirically to an equation of the same form in the hope that ultimately its constants can be evaluated in terms of equation (22).

For simplicity, the proposed isolation of units with shortening will be omitted from this part of the discussion and similarly the discussion will be confined to the range of lengths from rest length (L_o) to $2/3\,L_o$. All lengths L of the muscle will be expressed as a ratio to L_o and designated l, all loads P less than P_o, the maximum isometric tension developed at L_o, will be expressed as a ratio to P_o and designated p, and finally all velocities V will be measured as a ratio to V_o, the velocity of unloaded muscle and designated v.

Let L_o be a measure of the number of reactive pairs in any one contractile filament and P_oL_o the measure of the total number of reactive pairs in the muscle under no load. Assume that if a muscle contracts under a load p, the energy of activation of the contracting units must be increased by an amount cp. This extra energy mobilized into the contracting units can only be obtained at the expense of the total energy available. In the presence of the load p, the number of reacting units (n_p) with energies in excess of the average by an amount equal to or greater than cp will be less than the total number by the factor exp. $(-cp)$, hence equation (23).

$$n_p = P_oL_o \exp. (-cp) \tag{23}$$

Since only these activated molecules can shorten against the load, the maximum shortening will be proportional to n_p/P_o and this ratio will consequently specify the length-tension diagram. The velocity of shortening at any moment will be proportional to the number of activated molecules that have not yet undergone reaction, therefore the maximum velocity of shortening at any load p is also proportional to n_p/P_o. This is the same assumption used earlier.

Postponing discussion of the value of c for the moment, assume it has the average value of 3.0. For a load p, n_p reactive units have energies

Designate these reactive pairs as a–b, c–d, and e–f. In free shortening, the reaction would be random so that for example b–c or d–e might react, or even f might react with the reactive site of the next contiguous monomer and each pair reacting would degrade their equal energy content as shortening heat. The load may decrease the randomness of the reaction so that a and f, for example, could not react with units in their contiguous monomers. Assume that in the presence of the load, only the reactive pair c–d can shorten and only after the a–b or e–f pair has transferred energy in quantized fashion to c–d. Let c–d shorten under a load $P_o/3$. The pair c–d would then shorten and do one unit of work and liberate one unit of shortening heat, while the pair e–f would retain one unit of potential energy to restore the fraction expended as external work. This scheme, while no doubt naive, has the merit that it fulfills the statistical mechanical requirement that in the unit that actually shortens, the activation energy is only increased by the amount of the work done, while the work is being done, and only afterward does it gain energy from the potential energy present in e–f.

1. *Test of the Hypothesis*

Equation (23) does not allow for the postulated isolation of groups which must be taken account of in a test against real data. It was shown that if the series elastic element is taken as equal to 0.184 times the external shortening and is stretched by an amount p times its length, equation (23) becomes modified to equation (28).

$$S = L_o \exp. (-3p) (0.816 - 0.184p) \tag{28}$$

S specifies the maximum shortening for a load equal to p and hence the length tension diagram for all lengths equal to and less than L_o (Fig. 8). Similarly, with slight modification it expresses the force velocity relationship (Fig. 9). For the ancillary assumptions necessary to describe the length-tension diagram at lengths greater than L_o and also for the delta state length-tension diagram, the original analysis will have to be consulted.

2. *Summary*

The cycle and kinetics proposed account for the following properties of a muscle at rest length or at shorter lengths: (1) the virtually invariant magnitude of the maximum tetanic tension with temperature; (2) an invariant length-tension diagram; (3) the force velocity relation-

shortening is allowed to go to completion, the potential energy left in the structures is less than the work done. Since a muscle contracting under a load $p = 1/3$ shortens by approximately an amount equal to L_o/e, it is clear that if it shortens more than this under a lighter load, it fails to reserve enough potential energy to reverse the work done on the load and hence goes into the delta state where active relaxation is abolished.

In the original analysis, it was demonstrated that if the energy of the reaction which gave rise to "activation" or maintenance heat during contraction were used to restore the system during relaxation, it would be adequate to restore that fraction lost as shortening heat if the muscle did not shorten more than one-third of its length. At that time an "unknown" reaction was invoked to restore the fraction of energy lost as external work. The present analysis assumes that in the reversible range of contraction, the potential energy left in the structure restores that fraction of energy expended as external work and that the energy so lost could well be furnished by acceleration of the isothermal activation energy system in relaxation. One would thus assume that acceleration of this reaction would only occur under conditions where shortening was highly ordered, and that when shortening was great, disorder would prevent this acceleration.

In molar terms, P_oL_o of equation (24) would correspond to kTS/h of equation (22) and similarly to equation (27).

$$-cp = -\frac{\Delta F}{RT} - \frac{S\Delta p}{kT} \tag{27}$$

In the present hypothesis, the activation energy is actually concerned with restoration of the system and not "activation," hence the term $-\Delta F/RT$ in equation (27) probably is small. The assumption underlying equation (22) was that the energy of the contracting units had to be increased by an amount equal to the work done, yet equation (24) shows that the total excess energy mobilized in the reaction units was equal to three times the work done. This fact can only be reconciled with ordinary statistical mechanics by ascribing particular properties to the muscle machine. The most obvious property necessary is to have the contractile unit that is doing the shortening, and hence the work, activated by an amount of energy exactly equal to the work done.

Suppose that a monomer of a contractile filament had three substituents each of which had two reactive pairs lined up in linear array.

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ship; (4) the absence of fatigue in the contractile mechanism as judged by the fact that tension is maintained until excitation fails and the presence of fatigue in relaxation as judged by the fact that it is greatly prolonged by previous activity; (5) the prediction of active relaxation at lengths above 2/3 L_o and of the failure of active relaxation when the muscle shortens more than 1/3 L_o; (6) the prediction quantitatively (within the values experimentally determined) of the magnitude of shortening heat and the fact that it is practically constant per centimeter of shortening and is also independent of load or temperature or the time in a tetanus at which a muscle is allowed to shorten.

A plausible hypothesis is advanced to explain the series elastic element which accounts for its magnitude at least semi-quantitatively at rest lengths and predicts its increase with shortening. Finally, the analysis suggests that the length of the ultimate contractile monomer is some multiple of $1/e$ or 0.368. Since repeating units of from 350 A°. to 420 A°. units have been noted in X-ray studies of muscle (Schmitt *et al.*, 1947), a subunit 368 A°. long may not be too improbable.

Some criticism of the earlier analysis has been published. Two criticisms derive from a failure to understand the analysis and will not be discussed. The only experimental evidence against this cycle has been discussed under Section III, B, 2, c. Hill's further objection to the cycle on the grounds of the improbability of an isothermal reaction in relaxation is not tenable on two grounds: (1) in the Fenn-Hill cycle an isothermal cycle is implied in order to account for the work done; (2) as Needham (1950) emphasizes, a reaction having 100% efficiency in restoring the system in relaxation is by no means improbable, for it is known that during the first 3 or 4 min. after contraction and relaxation are over, under anaerobic conditions, glycogen is being broken down to lactic acid and creatine phosphate is being formed with 90% efficiency.

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CHAPTER VII

Thermodynamics of Muscle

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I. INTRODUCTION

The active state of muscle is characterized by the tendency of the tissue to contract. The motion resulting from this contractile tendency depends on both the relative length of the tissue and the mechanical constraints imposed upon it. For a wide variety of muscles, when shortening is restricted so that length-dependent parameters remain virtually constant, the velocity of contraction, v, is a function of the opposing mechanical load, p:

$$v = f(p) \tag{1}$$

This relation can also be written,

$$p = g(v) \tag{2}$$

where p may be thought of as the tension developed by the muscle when it shortens at rate v. Therefore dw/dt, the rate at which energy leaves the muscle as mechanical work, is

$$\frac{dw}{dt} = pv = vg(v) \tag{3}$$

Energy also leaves the muscle as heat. Although the heat flux is present in both passive and active muscle, it is considerably greater in active muscle. The elegant studies of Hill (1938) have shown that, in the

[1] The opinions expressed in this article are those of the author, and do not necessarily reflect the opinions of the Navy Department or the Naval service at large.

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x	distance
$\bar{F}_i$	$\delta F/\delta N_i$, the partial molal free energy of species i
$\bar{H}_i$	$\delta H/\delta N_i$, the partial molal enthalpy of species i
$\bar{S}_i$	$\delta S/\delta N_i$, the partial molal entropy of species i
$\bar{U}_i$	$\delta U/\delta N_i$, the partial molal internal energy of species i
$\bar{V}_i$	$\delta V/\delta N_i$, the partial molal volume of species i
$\bar{Y}_i$	$\delta Y/\delta N_i$, the partial molal Y
A,B,C	chemical reactions
$Ei(-u)$	$\int_{\infty}^{u} \frac{e^{-z}}{z}\,dz$, the exponential integral
F	free energy
H	enthalpy
M	number of T sites per cubic centimeter of muscle
N_i	mole numbers
P	pressure
Q	heat flow into the system
R	a chemical reaction
S	entropy
T	temperature
U	internal energy
V	volume
W	total work done by the system
Y	an extensive thermodynamic function
λ	$\int_{-\infty}^{\infty} k(x)\,dx$, the area under the $T-t$ interaction probability curve
ν	v/v_m, the speed of shortening normalized to unity
ξ	extent of chemical reaction
σ	$sv_m/2$

III. FIRST LAW OF THERMODYNAMICS; THE ENERGY BALANCE

When energy is converted from one form to another, the *total* energy is conserved. To apply this generalization, the first law of thermodynamics, to processes involving chemical transformations, consider a chemical reaction, A,

$$a_1A_1 + a_2A_2 + \ldots = \ldots + a_{s-1}A_{s-1} + a_sA_s, \tag{5}$$

proceeding to extent ξ. This means that the final mole numbers, N'_i,

active state, the rate of heat production, $-dQ/dt$, is a function of the velocity of contraction,

$$-\frac{dQ}{dt}=h(v) \tag{4}$$

In this chapter, the experimental observations embodied in Eqs. (1) and (2) will be analyzed thermodynamically and correlated with the kinetics of the chemical reaction which must be postulated as a source of energy for the contractile process.

II. SYMBOLS

The following notation will be used:

- a — constant relating the velocity of contraction to both the force developed by a muscle [Eq. (11)] and the rate of heat production [Eq. (14)]
- a_i — stoichiometric coefficients of reaction A. For reactants, $a_i < 0$; for products, $a_i > 0$
- b — constant in the relation between velocity of contraction and the force developed by a muscle [Eq. (11)]
- c_i — $N_i/\Sigma N_i$, the concentration of species i
- k_x — $T-t$ interaction probability per unit time
- l — distance between t sites on the thin filament
- n_x — probability of $T-t$ interaction when the transit of sites has proceeded from $-\infty$ to x
- π — $\int_{-\infty}^{\infty} n(x)\,dx$, the probability of $T-t$ interaction in a complete transit
- p — tension developed by contracting muscle
- p_o — isometric, tetanic tension
- $\dot{q}_o$ — the rate of heat production in isometric contraction
- r — ratio of the distance of a t site from the fixed end to the length of the folding region (in the folding model)
- s — length of a sarcomere
- t — time
- u — velocity of t sites relative to T sites
- v — velocity of contraction
- v_m — maximum velocity of contraction
- w — $W-P\Delta V$, the total work minus the work of isobaric expansion against the atmosphere

this when the second law of thermodynamics is discussed (Section VII).

In the present analysis, Eq. (10) will be used to calculate the extent of reaction from the energy exchanges associated with it.

$$\xi = \frac{1}{\widetilde{\Delta H}}(Q-w) \qquad (10')$$

In this application, $1/\widetilde{\Delta H}$ is simply a proportionality constant, and it is not necessary to consider its magnitude relative to w.

In the following, it will be assumed that $\widetilde{\Delta H}$ does not depend on ξ. Although this assumption is nearly always valid, it imposes upon the analysis a restriction which will be examined in Section VIII.

IV. CHEMICAL KINETICS OF MUSCULAR CONTRACTION

In muscular contraction, a chemical reaction is coupled to a mechanism capable of generating tension, thereby producing mechanical work. It will be assumed that the chemical change in this process can be described by a single parameter, the extent of reaction.[3] The most plausible reaction scheme with this property is diagrammed in Fig. 1.

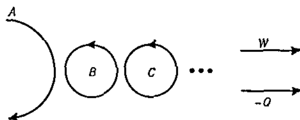


FIG. 1. Reaction scheme for the initial process of muscular contraction.

Reaction A is the *net* change in the chain of reactions leading to the development of tension. Reactions $B + (-B)$, $C + (-C)$, . . ., interposed between A and the system generating the tension, are assumed to be cyclic.[4] In other words, by the time the measurements of heat

[3] For support of this assumption, see the discussion of the recovery process (Section VI).

[4] The turnover of intermediate reactions B, C, . . . , can be studied chemically with isotopic methods. They can also be examined by breaking the reaction chain at some point either with a chemical inhibitor or a change in the physical parameters, pressure and temperature. For example, the latter technique has been used in an attempt to prevent reactions A, B, . . ., from reversing reactions more intimately connected with tension development (Mommaerts, 1954; Fleckenstein, 1954). Muscles, stimulated at normal temperature, were frozen as quickly as possible after the development of tension and subjected to chemical analysis. If the time sequence of reactions is from right to left, the net chemical change should depend on the interval between tension development and freezing. By varying this interval, the links in the chain can, in principle, be sorted out.

are related to the initial mole numbers, N_i, according to the equation

$$N_i' = N_i + a_i \xi \tag{6}$$

where the stoichiometric coefficients, a_i, are positive for products and negative for reactants. If ΔU is the change in internal energy when the extent is ξ, the first law of thermodynamics requires that

$$\Delta U = \xi \widetilde{\Delta U} = Q - W \tag{7}$$

where $\widetilde{\Delta U}$ is the change in internal energy for unit extent,[2] Q is the heat flow *into* the system, and W is the work done *by* the system. Many processes, including muscular contraction, take place under the constant pressure, P, of the environment. If the volume change accompanying such an isobaric process is ΔV, an amount of energy $P\Delta V$ necessarily goes into the work of expansion. It is convenient to dissect this expansion work from the total work,

$$W = w + P\Delta V \tag{8}$$

where, by definition, w is the work done by the system in excess of $P\Delta V$. If $\widetilde{\Delta V}$ is the change in volume of the system when the extent of reaction is unity, Eqs. (7) and (8) can be combined:

$$\xi(\widetilde{\Delta U} + P\widetilde{\Delta V}) = Q - w \tag{9}$$

This relation can be written more compactly in terms of the enthalpy, $H \equiv U + PV$,

$$\xi \widetilde{\Delta H} = Q - w \text{ (isobaric process)} \tag{10}$$

The quantity $-\widetilde{\Delta H}$ is the thermal energy *released* by reaction A when the extent is unity and $w = 0$. It is the heat measured when the reaction is carried out in an isobaric calorimeter, and can be either positive (exothermic reaction) or negative (endothermic reaction).

Sometimes Eq. (10) is interpreted to mean that $-\xi\widetilde{\Delta H}$ is the energy released during the chemical reaction, and that this energy is manifested either as work, w, or as heat, $-Q$. This is not correct since there are isothermal reactions for which $Q > 0$ and the work coupled from the reaction *exceeds* the quantity $-\xi\widetilde{\Delta H}$, the excess energy being drawn from the thermal energy of the environment. More will be said about

[2] In general, the superscript curl in $\widetilde{\Delta X}$ will denote the change in X for unit extent of reaction.

The release of chemical energy in reaction A is reflected in work and heat production. The experiments from which these quantities are calculated in the present analysis are summarized in Figs. 2 and 3. Both figures are taken from the classic paper of Hill (1938) on the dynamic constants and the heat exchanges of contracting frog sartorius muscle. Figure 2 relates the load, p, and velocity of shortening, v, in isotonic contraction. Since the velocity is constant, p is also equal to the tension developed by the muscle. The circles are experimental; the curve is calculated from the empirical equation

$$(p + a)v = b(p_o - p) \tag{11}$$

where a and b are characteristic constants of the muscle. The intercept on the abscissa is the tetanic tension, p_o, a function of the length of the mucle. The intercept on the ordinate is the maximum velocity, $v_m = bp_o/a$. In the following, it will be assumed that the muscle is approximately at natural body length and that shortening is small enough so that changes in length-dependent functions, like tetanic tension, can be neglected. In other words, although the muscle shortens, it is assumed that the length remains in the region of the broad maximum of the length-tension curve (Volume II, Chapter VI). Then the tension can be written as a function of the velocity of contraction,

$$p = \frac{bp_o - av}{v + b} \tag{12}$$

Therefore the rate of doing work, $\dot{w} = dw/dt$, or the *power* of the muscle, is

$$\dot{w} = pv = \frac{v(bp_o - av)}{v + b} \tag{13}$$

This relation shows that the speed of shortening controls the rate at which mechanical energy leaves the muscle.

Energy also leaves the muscle as heat. Figure 3 summarizes experiments in which $-Q$, the total heat produced by tetanically stimulated muscle, was measured before, during, and after shortening at different velocities. It is seen that the speed of shortening governs this process also. In 3(a) a muscle 29.5 mm. in length was released at 1.2 sec. after the beginning of stimulation and allowed to shorten different distances under a constant load. Curves A, B, C, and D are for shortening 0, 1.9,

and work have been completed, each of the intermediate reactions B, C, . . . , will have been balanced by the reverse reactions $-B$, $-C$, ...

Such a series of linked reactions has the following properties. In the first place, the amount of chemical energy made available is proportional to the extent of reaction A. Thus the rate at which chemical energy is made available in the active state of muscle depends on the rate of reaction A. Secondly, since the chemical reactions are coupled, any reaction in the chain can be rate limiting. Thus if reaction C, say,

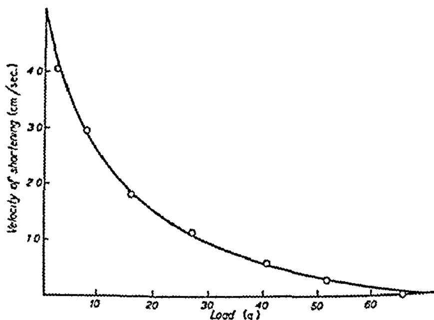


FIG. 2. Relation between velocity of shortening and the mechanical load in isotonic contraction. From Hill, 1938, p. 177.

were coupled to the contraction mechanism, and if the turnover of C were limited by some property of the contraction mechanism, this property would also control the rate of reaction A, since A can proceed no faster than C. In this respect, reaction A is comparable to the mainspring of a timepiece. *The energy delivered by the mainspring* is limited by the motion of the last wheel in the chain of gears linking it with the escapement mechanism. Thirdly, thermodynamic properties of the cyclic reactions are not reflected in the energy fluxes. Thus, if turnover is complete for the reaction most intimately connected with the development of tension, the following thermodynamic equations will be unaffected by the *energetics* of this reaction.

the velocity of contraction. This requires the rate of heat production to be a linear function of the velocity of contraction,

$$-\dot{Q} = -\frac{dQ}{dt} = \dot{q}_o + av \tag{14}$$

where $\dot{q}_o$ is the rate of heat production is isometric contraction. The constant a is dimensionally and numerically the same as the a in the force velocity relation [Eq. (11)]. This means that the rate of work and heat production in excess of the rate for isometric contraction [the left-hand side of Eq. (11)] is a linear function of the load on the muscle.

In the experiments shown in Fig. 3(c), the muscle was released at various times after the start of stimulation and allowed to shorten a constant distance under a constant load. Curve K is an isometric contraction. Since the initial slopes of N, M, and L, as well as the final displacements from curve K, are essentially the same, the time of release does not affect the validity of Eq. (14).

These heat measurements can be summarized by saying that the heat flux from stimulated muscle depends on the speed of shortening, v. A contribution proportional to v is superimposed on the heat of activation, the basal rate of heat production for stimulated muscle constrained to remain at constant length.

The empirical expressions for w and $\dot{Q}$ can be combined with the differentiated form of Eq. (10),

$$\dot{\xi}\,\Delta\tilde{H} = \dot{Q} - \dot{w} \tag{15}$$

to derive an expression for the rate of reaction A, $\dot{\xi} \equiv d\xi/dt$, as a function of the velocity of contraction. With Eqs. (13) and (14), Eq. (15) becomes, after algebraic manipulation,

$$-\dot{\xi}\,\Delta\tilde{H} = \frac{\dot{q}_o b + \left[q_o + b(a + p_o)\right] v}{b + v} \tag{16}$$

Thus the chemical reaction rate, $\dot{\xi}$, is a hyperbolic function of the mechanical reaction rate, v. When v is zero, the corresponding reaction rate, $\dot{\xi}_o$, is

$$\dot{\xi}_o = -\dot{q}_o/\Delta\tilde{H} \tag{17}$$

When v is the maximum shortening rate, $v_m = bp_o/a$, corresponding to

3.6, and 5.2 mm., respectively. During shortening the rate of heat production increases relative to the rate for isometric contraction. Since the final parts of curves B, C, and D are nearly parallel to A, the rate of heat production in the shortened state is essentially the same as in the corresponding isometric state.[5] The displacements of B, C, and D from curve A, that is, the total extra heat due to shortening, are proportional to the amount of shortening.

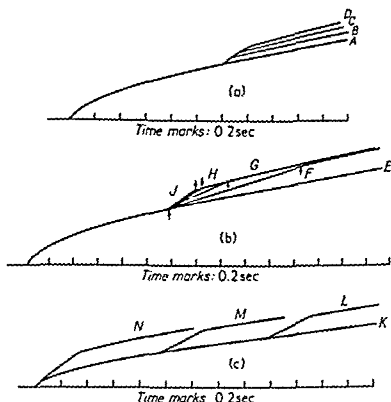


FIG. 3. Total heat production (ordinate) as a function of time. Tetanically stimulated muscle at 0°C. See text for the experimental conditions. From Hill, 1938, p. 159.

The heat from the same muscle shortening a constant distance under increasing loads is shown in J, H, G, and F, respectively, of Fig. 3(b). The muscle was released at 1.2 sec.; the end of shortening is indicated by arrows. Curve E is an isometric contraction. Since J, H, G, and F are displaced from the isometric contraction by the same amount of heat, the total heat in excess of the isometric heat does not depend on

[5] Actually, $-\dot{Q}$ for $v = 0$ becomes somewhat greater when the muscle is less than "normal body length."

With (19) and (20) in (18), $\dot{\xi}_m = 5\dot{\xi}_o$, so that the reaction rate for unloaded contraction is five times greater than that for isometric contraction. A form of (16) particularly convenient for computations follows from substitution of the empirical relations (19) and (20) and introduction of the reduced velocity,

$$\nu = v/v_m = va/bp_o \tag{21}$$

as a variable:

$$\dot{\xi} = \dot{\xi}_o (1 + 24\nu) / (1 + 4\nu) \tag{22}$$

This hyperbolic dependence of the driving reaction rate on the velocity of contraction, shown in Fig. 4, is a requirement of any mechanochemical model, regardless of the detailed mechanism of the contractile process.

The extent of the driving reaction for a given *distance* of contraction is also of interest. Since

$$\frac{d\xi}{dx} = \frac{1}{\nu} \frac{d\xi}{dt} \tag{23}$$

this quantity is simply the ratio of the ordinate to the abscissa of Fig. 4 and is plotted as the dotted curve. If the driving reaction A were coupled directly to the displacement of the muscle, like a rack and pinion device, $d\xi/dx$ would be independent of the shortening speed. This mechanism is untenable since $d\xi/dx$ increases with decreasing velocity, showing that, for a given amount of shortening, reaction A proceeds to a greater extent when the muscle moves slowly rather than rapidly. Rather, the mechanochemical coupling appears to have properties more like a fluid drive permitting slippage between input and output shafts.

V. MODELS OF THE KINEMATICS OF CONTRACTION

The kinetics illustrated in Fig. 4, in which a reaction rate depends on the shortening velocity, are probably a clue to the mechanism of the contraction process. Therefore, two simple models having an anatomical basis and manifesting these kinetic properties will be described. It will be shown that kinetics closely approximating those derived from thermodynamic analysis of the empirical relations of Hill can be deduced from models in which a reaction rate depends on the relative positions of two moving sites.

The physical basis for these models is described in Volume I, Chapter

unloaded contraction [Eq. (11)], the chemical reaction rate increases to $\dot{\xi}_m$,

$$\dot{\xi}_m = \dot{\xi}_o(1 + bp_o/q_o) \tag{18}$$

This expression can be evaluated numerically using the empirical observations (Hill, 1939b)

$$\dot{q}_o = ab \tag{19}$$

and

$$p_o = 4a \tag{20}$$

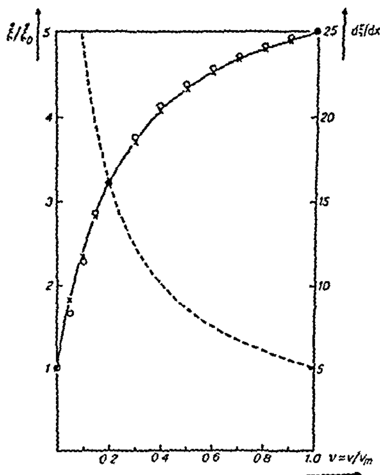


Fig. 4. *Smooth curve:* Relative rate of reaction, $\dot{\xi}/\dot{\xi}_o$, as a function of the reduced velocity of contraction, $\nu = v/v_m$. Corresponding kinetics for a sliding model are plotted as *circles*; kinetics for a folding model are plotted as *crosses* (Section V). Both models are scaled to coincide with the smooth curve at $\nu = 0.2$ and $\nu = 1.0$ *(closed circles)*.

Dotted curve: Relation between the extent of reaction for a unit distance of contraction, $d\xi/dx$, and the velocity of contraction.

on the relative position of the sites (Fig. 6). Also, in the course of the transit of a t site past a T site, the interaction event (a) either does or does not happen, and (b) can happen only once. Now, continuing with the example in which t is a binding site for a molecule and T is the *site of an enzyme catalyzing* the hydrolysis of this molecule, as t moves past T the probability of hydrolysis will be greater, the closer the sites. In a transit of the sites, the bound molecule either does or does not suffer hydrolysis, and the reaction can happen only once. If this reaction is the rate limiting step in the chain (Fig. 1), it can control the rate of the reaction sequence. In this case, the interaction rate can be equated to $d\xi/dt$.

To derive the kinetics of the interaction process, consider a pair of sites moving past each other with relative velocity u. Suppose the displacement of t relative to T is x. Then the probability that interaction will have occurred already is n_x. The probability δn that interaction will occur in the next element of time, δt, is the product of $(1-n_x)$, the probability that interaction has not occurred yet, and $k(x)\ \delta t$, the *a priori* probability factor for interaction,

$$\delta n = (1 - n_x) k_x\, \delta t \tag{24}$$

Since δx, the distance traversed in the element of time δt, is $u\delta t$,

$$\delta n = (1 - n_x) k_x\, \delta x / u. \tag{25}$$

Integration across the entire transit gives n, the probability for interaction in a complete transit at velocity u,

$$n = 1 - e^{-\lambda/u} \tag{26}$$

where $\lambda = \int_{-\infty}^{\infty} k_x dx$, the area under the interaction probability curve. When the velocity, u, is zero, the probability for interaction, n, is unity. As u increases, the value of n decreases, since in the more rapid transit there is less time available for interaction. Finally, when u is infinite, n vanishes. These interactions are qualitatively similar to reactions in living muscle, where $d\xi/dx$, the extent of reaction per unit of shortening, also decreases with increasing velocities (Fig. 4).

It is seen that n does not depend on the particular shape of the interaction probability curve as long as the enclosed area remains constant. In particular, rather than the symmetric function chosen for illustration, k_x might be asymmetric.

VII. According to this view, skeletal muscle is made up of two sets of interdigitating filaments, represented in Fig. 5 by thick and thin horizontal lines. When muscle contracts, the thin filaments appear to be drawn into the matrix of the thick filaments. In this change, there is relative motion of thick and thin filaments. Now suppose the rate of one of the reactions linked stoichiometrically with the driving reaction A (Fig. 1) depends on the position of sites on the thin filaments relative

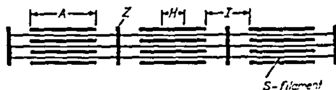


Fig. 5. Diagram of a muscle fibril. See Volume I, Chapter VII, for discussion of A, H, and I regions, Z line, and S filament. From Huxley, 1957, p. 261.

to complementary sites on the thick filaments. Then the kinetics of this reaction will depend on the speed with which the thin and thick filaments move past each other, linking the speed of shortening to the rate of the driving reaction.

To characterize such a geometrically constrained reaction, consider Fig. 6. The reaction is supposed to involve two components, one at the t site on the thin filament and the other at the T site on the thick

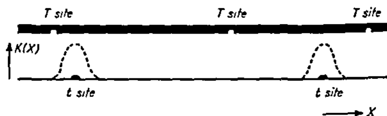


Fig. 6. Thick and thin filaments of a muscle fibril. See text for description.

filament. An example of such a mechanism is the hydrolysis of a substrate bound to the t site by an enzyme located at the T site. As the sites pass each other, there is a certain probability that the substrate will be hydrolyzed through interaction with the enzyme. However, since the following analysis does not depend on the specific chemical mechanism, the reaction between a molecule at the T site with its complement at the t site will be called "T—t interaction."

It will be assumed that the T—t interaction has the following characteristics. The interaction probability per unit time, k_x, depends

Consider the sliding model first. The velocity, u of t sites on the thin filaments relative to T sites on the thick filaments is the same along the length of the filaments. This velocity is related to the rate of shortening of the intact muscle, v (measured in muscle lengths per second), by the expression

$$u = sv/2 \tag{27}$$

where s is the sarcomere length. If the distance between t sites is l, each T site will be presented with u/lt sites per unit of time. Therefore, the interaction rate per cubic centimeter of muscle is Mun/l, where M is the number T of sites per cubic centimeter of muscle. Assuming the interaction rate limits the rate of chemical reaction, and taking n from (26),

$$\xi - \xi_0 = \frac{Mu}{l}(1 - e^{-\lambda/u}) \tag{28}$$

Combination of (27) and (28) gives the rate of chemical reaction as a function of the speed of shortening of the ends of the muscle.

In the folding model, the ends of the thin filament are assumed to be fixed relative to the thick filaments. Upon stimulation, the thin filaments are supposed to fold, reducing the linear distance between the fixed ends, thereby pulling the thick filaments closer together (Fig. 7). In the following it will be assumed that (a) the folding is linear, as in a simple spring, and (b) the region of the thin filament in which the folding takes place is fixed in length and is initially interdigitated with the thick filaments.

The velocity of t sites relative to the T sites will be zero at the fixed end of the thin filament and u at the part of the folding region closest to the I band, where, as in the sliding model, the shortening velocity of the sarcomere is $2u$. Therefore, if r is the ratio of the distance of a given t site from the fixed end to the length of the folding region, its relative velocity will be ru. A T site at the corresponding part of the thick filament will suffer ru/l transists, where l is the distance between t sites. Therefore, the reaction rate per cubic centimeter of muscle in the folding model of contraction is

$$\dot{\xi} = \frac{Mu}{l}\int_0^1 r(1 - e^{-\lambda/ur})\, dr \tag{29}$$

[a] For the sliding model, an alternative derivation of the relation between the rate of chemical reaction and the speed of shortening can be found in the excellent analysis of A. F. Huxley (1957).

Before this analysis can be extended to a contracting muscle, the distribution of sites along the thick and thin filaments and the relative number of these filaments must be known. It will be assumed that sites are distributed with uniform linear density along each set of filaments. The average spacing on the thick and thin filaments need not be the same. Each $T - t$ interaction is supposed to be independent of interactions during previous transits. Thus the interaction between a t site and the nearest T site does not depend on whether there was interaction when it passed the preceding T site upstream. Also, the interaction probability function k_x is supposed to be sufficiently narrow to isolate sites from their neighbors on the same filament.

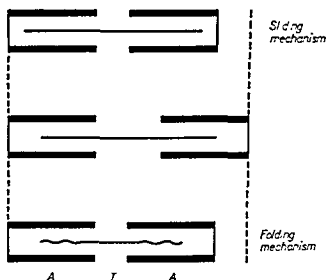


FIG. 7. Sliding and folding contraction mechanisms. *Center*; sarcomere before contraction; *Top* and *bottom*; sarcomere after contraction.

The relative velocity of sites along the two sets of filaments will depend on the relative motion of filaments during the contraction process. Current models for contraction can generally be classed as either *sliding* or *folding* models (Fig. 7). In the sliding model, the contraction force is supposed to arise from interaction between the thick and thin filaments. In the folding model, the force stems from a change in the elasticity of the thin filaments. This requires the ends of the thin filaments to be fixed relative to the thick filaments during the initial process. In both models, contraction is accompanied by a shortening of the I band, the A band width remaining constant, in agreement with the microscopical evidence.

initial hypothesis that interaction between filaments in relative motion controls the rate of the driving reaction. However, since the kinetic curves for both sliding and folding models can be made virtually indistinguishable from each other, shape alone cannot be rallied as evidence for one or the other contraction mechanism. If the distribution of the interaction sites were independently known, discrimination between the two models could be made on the basis of the different predicted values of M/l (Table I). Also, the nature of the heat of activation, a term presently added arbitrarily to both sliding and folding models to make them agree with experiment, might be revealed.

VI. THE RECOVERY PROCESS

Since a muscle can exert tension over and over again, there clearly is some mechanism for reversing the chemical processes accompanying contraction. On the molecular level, this means that reaction A must be reversed. Thus a cycle of muscular activity divides naturally into two phases (Fig. 8). In the *initial process*, catalyzed by stimulation, the driving reaction A proceeds to some extent and energy leaves the

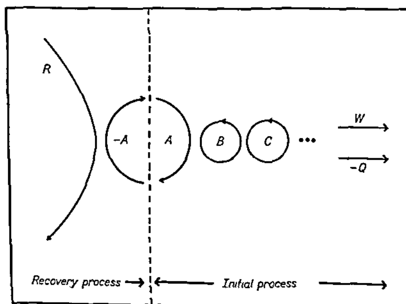


FIG. 8. Reaction scheme for a cycle of muscular activity.

system as work, w, and heat, $-Q$. The total energy efflux, $w-Q$, is called the *initial energy*. The initial process is followed by a slower *recovery process* in which A is reversed through coupling with another reaction, R.

where, as before, it is assumed that $\dot{\xi}$ is limited by the rate of $T-t$ interaction. Carrying out the integration,

$$\dot{\xi} = \frac{Mu}{2l}\left(1 - e^{-\lambda/u} + \frac{\lambda}{u}\left[e^{-\lambda/u} + \frac{\lambda}{u}\,Ei\left(-\frac{\lambda}{u}\right)\right]\right) \tag{30}$$

where $Ei\left(-\frac{\lambda}{u}\right)$ is the exponential integral $\int_{\infty}^{\lambda/u} \frac{e^{z}}{z}\,dz$. Comparing (30) with the corresponding expression for the sliding model (28), two differences are apparent: the square bracketed term within the parentheses and the factor of 1/2 without. The latter appears because the *average* relative velocity for site transit is $u/2$ for the folding model, and u for sliding model.

The kinetics predicted by the sliding and folding model of contraction could be compared with the hyperbolic function of contraction velocity [Eqs. (16) and (22)] derived from the Hill equations for work and heat production if the quantities M/l and λ were known. However, even without independent values of M/l and λ, the two models can be compared by *selecting* these constants to make the kinetics agree with the hyperbola at two different relative velocities. Then the values of ξ for the other relative velocities can be calculated and compared with the hyperbola. The results of this procedure are shown in Fig. 4. Both models were fitted to the hyperbola at relative velocities of 0.2 and 1.0 (closed circles) by selecting appropriate values for M/l and λ (Table I). The calculated values of $\dot{\xi}$ for *both* the sliding model

TABLE I

PARAMETERS OF THE SLIDING AND FOLDING MODELS[a]

Model	$\frac{M}{l\dot{\xi}_0}\sigma$	$\frac{1}{\lambda}\sigma$
Sliding model: equations (27) and (28)	13.3	2.79
Folding model: equations (27) and (30)	29.9	5.68

[a] In both models the parameters $M\sigma/l\dot{\xi}_0$ and σ/λ, where $\sigma = sv_m/2$, were selected to fit $d\xi/dt$ at relative velocities 0.2 and 1.0 (closed circles in Fig. 4) to the values derived from Hill's relations.

(open circles) and the folding model (crosses) are then seen to closely approximate the hyperbolic curve. This good agreement supports the

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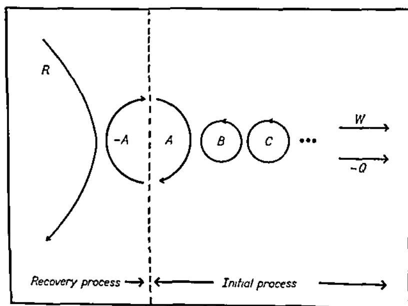


FIG. 8. Reaction scheme for a cycle of muscular activity.

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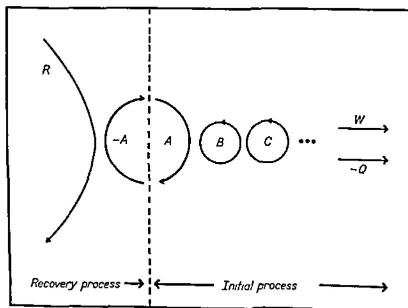


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This expression, which can be taken as the definition of ΔS for a particular change of state, shows that the entropy change is related to the tendency of the temperature of a system to change during reaction. Thus if $\Delta S > 0$, heat flows from the environment into the system to maintain isothermal conditions, implying that the temperature tends to decrease in the reaction. Conversely, if $\Delta S < 0$, the temperature of the system tends to increase in the course of reaction. These temperature changes could be observed if the reactions were carried out adiabatically rather than isothermally. On the molecular level, the temperature changes are related to the change in quantum mechanical properties of the reacting molecules.

It should be stressed that the heat can be calculated from the entropy change only when there are suitable coupling mechanisms for extracting the maximum amount of work from the reaction. Otherwise, the heat gained by the system will be less than Q_{max}, given by Eq. (34). It is as though the difference between the maximum work and the actual work is degraded into heat which augments the heat due to the entropy change.

It is convenient to construct a thermodynamic function that accounts for the ability of reactions to do work through changes in entropy. The energy available for work in an isobaric reaction, w_{max}, is less than W_{max} [Eq. (33)] because of the expansion work, $P\Delta V$. Thus

$$w_{max} = W_{max} - P\Delta V = T\Delta S - \Delta U - P\Delta V \tag{35}$$

The free energy, F, is defined as

$$F \equiv U + PV - TS = H - TS \tag{36}$$

Comparing (35) and (36), and taking T and P as constant,

$$w_{max} = -\Delta F \text{ (isothermal, isobaric)} \tag{37}$$

The maximum work that can be coupled from an isothermal, isobaric reaction is the decrease in the free energy accompanying the reaction. This important relation will be used in the definition of the thermodynamic efficiency of muscular contraction (Section IX).

VIII. THERMODYNAMICS OF SOLUTIONS

It was remarked in Section III that $\Delta\tilde{H}$, the enthalpy change for unit extent of reaction, can usually be treated as a constant quantity. However, this is not the case for $\Delta\tilde{F}$, the corresponding free energy change. The nature of this important difference will now be examined.

In general, thermodynamic functions can be divided into two classes. The first class includes quantities like volume, energy, and entropy, which are proportional to the total amount of matter in the system. These *extensive quantities*, Υ, depend on the composition of the system,

$$\Upsilon = \Upsilon(\mathcal{N}_i) \tag{38}$$

and are homogeneous functions[1] of degree one:

$$\alpha\Upsilon = \Upsilon(\alpha\mathcal{N}_i) \tag{39}$$

where α is an arbitrary constant. The second class contains quantities that depend on the mole ratios but are independent of the total mass of the system. These concentration dependent functions, such as the density, are called *intensive quantities*.

Several mathematical consequences of Eq. (39) have important thermodynamic implications. One is that

$$\Upsilon = \Sigma\mathcal{N}_i\overline{\text{Y}}_i \tag{40}$$

where $\overline{\text{Y}}_i \equiv \delta\Upsilon/\delta\mathcal{N}_i$, the partial molal Υ. This means that any extensive thermodynamic quantity is a linear function of the mole numbers. The quantity $\overline{\text{Y}}_i$ can be thought of as the contribution of a unit amount of species i to the value of Υ for the system.

A second mathematical consequence of Eq. (39) follows from the fact that any *k–th* derivative of a homogeneous function of degree h is a homogenous function of degree h–k. In particular, since Υ is a homogeneous function of degree one, the partial molal Υ, a first derivative, is homogeneous of degree zero. Thus

$$\overline{\text{Y}}_i = \overline{\text{Y}}_i(\mathcal{N}_i) = \overline{\text{Y}}_i(\alpha\mathcal{N}_i) \tag{41}$$

Letting $\alpha = 1/\Sigma\mathcal{N}_j$

$$\overline{\text{Y}}_i = \overline{\text{Y}}_i(\text{c}_i) \tag{42}$$

where concentration, c_i, is defined as $\mathcal{N}_i/\Sigma\mathcal{N}_j$. Thus the partial molal quantities $\overline{\text{Y}}_i$ are intensive properties of the system.

The change in an extensive thermodynamic function upon chemical reaction can be calculated from Eq. (40). The mole numbers change according to Eq. (6). Thus after extent of reaction ξ the value of Υ is

$$\Upsilon + \Delta\Upsilon = \Sigma(\mathcal{N} + a_i\xi)\ (\overline{\text{Y}}_i + \Delta\overline{\text{Y}}_i) \tag{43}$$

[1] A function $f(x, y, z\ldots)$ is a homogeneous function of degree h if $f(\alpha x, \alpha y, \alpha z, \ldots) = \alpha^h f(x, y, z, \ldots)$.

where $\Delta\bar{Y}_i$ is the change in $\bar{Y}_i$ due to the change in concentrations. Subtracting Eq. (40) from (43) gives $\Delta\Upsilon$, the change in Υ resulting from the reaction,

$$\Delta\Upsilon = \xi\Sigma a_i(\bar{Y}_i + \Delta\bar{Y}_i) + \Sigma\mathcal{N}_i\Delta\bar{Y}_i \tag{44}$$

Thus $\Delta\Upsilon$ has two components. The first, proportional to the extent of reaction, is simply due to the change in mole numbers of the reacting nolecules. It stems from the fact that Υ is an extensive quantity and depends on the total amounts of the various components. The second contribution to $\Delta\Upsilon$ is due to the concentration dependence of the partial molal quantities. The terms in the sum $\Sigma\mathcal{N}_i\Delta\bar{Y}_i$ can be thought of as changes in the amount each molecule of species i contributes to the total Υ.

The influence of concentration on $\bar{Y}_i$ depends on the nature of Υ. Experimentally, the following are found to be good approximations for dilute solutions:

$$\Delta\bar{U}_i = 0 \tag{45}$$

$$\Delta\bar{V}_i = 0 \tag{46}$$

$$\Delta\bar{S}_i = R\log c_i'/c_i'' \tag{47}$$

where c_i' and c_i'' are the initial and final concentration, respectively.

Since $\Delta\bar{U}_i + P\Delta\bar{V}_i = \Delta\bar{H}_i$ and $\Delta\bar{H}_i - T\Delta\bar{S}_i = \Delta\bar{F}_i$,

$$\Delta\bar{H}_i = 0 \tag{48}$$

$$\Delta\bar{F}_i = RT\log c_i''/c_i' \tag{49}$$

Therefore, in this approximation, $\Delta\bar{U}$, $\Delta\bar{V}$, and $\Delta\bar{H}$ are independent of concentration, while $\Delta\bar{S}$ and $\Delta\bar{F}$ depend on concentration in a calculable way. These expressions define an ideal solution, which bears the same relation to a real solution as an ideal gas to a real gas.[9] Although departures from these relations become noticeable with increasing concentration, their validity will be assumed in the remainder of this section.

[9] In sufficiently dilute solution, the energy and volume of a solute molecule will depend only on interaction with solvent molecules. Departures from Eqs. (45)-(49) at higher concentration are due to increasing solute-solute interaction.

In contrast, statistical considerations show that the entropy at a given temperature depends on a geometrical factor, the volume available to each molecule. Since the same statistics can be applied in both cases, Eq. (47) for a solute in dilute solution is the same as the entropy change upon isothermal expansion of a perfect gas.

$\Upsilon = H$. Combining Eqs. (44) and (48),

$$\Delta H = \xi \Sigma a_i \overline{\text{H}}_i \tag{50}$$

where the quantities $\overline{\text{H}}_i$ are the partial molal enthalpies of the initial state. Thus the change in enthalpy is proportional to the extent of reaction, regardless of the resultant concentration change (as was assumed in Section III). Furthermore, since the enthalpy of dilution is taken to be zero [Eq. (48)], it can be stated that

$$\Delta H = \xi \Sigma a_i \overline{\text{H}}_{io} \tag{51}$$

where, in this case, $\overline{\text{H}}_{io}$ refers to a state with standard concentrations, c_{io}. Thus the change in enthalpy for unit extent of reaction does not depend on the initial concentrations of reactants. From Eqs. (50) and (51), the enthalpy change for unit extent of reaction, $\widetilde{\Delta H}$, is

$$\widetilde{\Delta H} = \Sigma a_i \overline{\text{H}}_i = \Sigma a_i \overline{\text{H}}_{io} \tag{52}$$

$\Upsilon = F$. Considering Eq. (44), a simple expression for the free energy change can be written only when the extent is restricted so that the concentrations do not change significantly in the course of reaction. In this case, $\Delta \overline{\text{F}}_i = 0$ and

$$\Delta F = \xi \Sigma a_i \overline{\text{F}}_i \tag{53}$$

where the quantities $\overline{\text{F}}_i$ are the partial molal free energies *at the existing concentrations*.

It is customary to tabulate values of $\widetilde{\Delta F}_o$, the free energy change for a unit extent of reaction, in a standard state of the system at concentrations c_{io},

$$\widetilde{\Delta F}_o = \Sigma a_i \overline{\text{F}}_{io} \tag{54}$$

Combining (53) and (54),

$$\Delta F = \xi \left[\widetilde{\Delta F}_o + \Sigma a_i (\overline{\text{F}}_i - \overline{\text{F}}_{io}) \right] \tag{55}$$

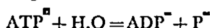
Using (49) to evaluate the second term in the brackets,

$$\Delta F = \xi \left[\widetilde{\Delta F}'_o + RT \log \Pi \, c_i^{a_i} \right] \tag{56}$$

where the numerical value of the constant $\widetilde{\Delta F}'_o \equiv \widetilde{\Delta F}_o - RT \log \Pi c_{io}^{a_i}$ depends on the choice of standard states. The free energy change for unit extent of reaction, $\widetilde{\Delta F}$, is

$$\widetilde{\Delta F} = \widetilde{\Delta F}'_o + RT \log \Pi c_i^{a_i} \tag{56'}$$

It is seen that, unlike ΔH, the value of ΔF in a reacting system can not be estimated unless the existing concentrations, c_i, are known. Since Eq. (56′) permits calculation of the free energy change of a reaction at the existing concentrations from ΔF_0, the free energy change in the standard state, it is one of the most important relations in chemical thermodynamics. For example, consider the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and phosphate (P), a reaction frequently implicated in muscular contraction. The standard free energy change for the reaction



$$ATP^{\equiv} + H_2O = ADP^{-} + P^{=}$$

is —5.9 Cal. per mole (Morales, 1956). With Eq. (56), it can be calculated that the change in free energy for hydrolysis at the concentrations of ATP, ADP, and P actually found in frog muscle is —9.4 Cal. per mole. Numerically, this value for ΔF is nearly two times greater than the corresponding value of ΔH (Podolsky and Morales, 1955).

IX. EFFICIENCY OF MUSCULAR CONTRACTION

The *efficiency* of a process capable of coupling work to a load is the ratio of the work actually done to the maximum amount that could have been done. The maximum amount of work that can be derived from a process depends, of course, on the mechanism of energy conversion. In a mechanical process, a given potential energy is made available for conversion into work. The fraction of this energy not appearing as work is manifested as heat. Thus the maximum work a mechanical system can do is $W - Q$, and the *mechanical efficiency* is

$$E_{\text{mech}} = \frac{W}{W - Q} \tag{57}$$

Clearly, since a mechanical process is characterized by the fact that $Q \leq 0$, that is, the flow of heat is always from the system to the environment, its mechanical efficiency can never exceed unity.

Consider next a mechanochemical system in which work is derived from a chemical reaction proceeding to extent ξ. According to the second law of thermodynamics, the maximum work that can be derived from an isothermal, isobaric reaction is $-\Delta F = -\xi \Delta \tilde{F}$. Therefore the *thermodynamic efficiency* is defined as

$$E_{\text{thermo}} = \frac{w}{-\xi \Delta \tilde{F}} \tag{58}$$

This can also be written in terms of the rate of work production, $\dot{w}$, and the speed of the chemical reactions, $\dot{\xi}$,

$$E_{\text{thermo}} = \frac{\dot{w}}{-\dot{\xi}\widetilde{\Delta F}} \tag{59}$$

In muscular contraction, although both $\dot{w}$ and ξ can be estimated as functions of the speed of shortening, the thermodynamic efficiency of the initial process cannot be calculated because the value of $\widetilde{\Delta F}$, the free energy change associated with the driving reaction, is not known.

Since only heat and work measurements are involved, mechanical efficiencies are sometimes calculated for mechanochemical processes when the thermodynamic properties of the driving chemical reaction are not known. Therefore, it is of interest to examine the relation between E_{mech} and E_{thermo} At the outset, it can be seen that mechanical efficiency is not an appropriate function for chemical reactions since Q can be negative and W_{mech}, as defined in Eq. (57), can exceed unity.

Dissecting the expansion work from the total work done in a reaction, Eq. (57) can be written

$$E_{\text{mech}} = \frac{w}{w-Q} \tag{60}$$

where $w = W - P\Delta V$. With Eq. (10),

$$E_{\text{mech}} = \frac{w}{-\xi\widetilde{\Delta H}} \tag{61}$$

In terms of the rates of doing work and of reaction,

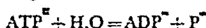
$$E_{\text{mech}} = \frac{\dot{w}}{-\dot{\xi}\widetilde{\Delta H}} \tag{62}$$

Comparing Eqs. (58) and (61),

$$E_{\text{thermo}} = \frac{\widetilde{\Delta H}}{\widetilde{\Delta F}} E_{\text{mech}} \tag{63}$$

and the two efficiency functions are proportional. The factor $\widetilde{\Delta H}/\widetilde{\Delta F}$ stems from the fact that the energy made available by an isothermal, isobaric chemical change is not $(w-Q) = -\Delta H$ but $-\Delta F = -(\Delta H - T\Delta S)$.

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is —5.9 Cal. per mole (Morales, 1956). With Eq. (56), it can be calculated that the change in free energy for hydrolysis at the concentrations of ATP, ADP, and P actually found in frog muscle is —9.4 Cal. per mole. Numerically, this value for ΔF is nearly two times greater than the corresponding value of ΔH (Podolsky and Morales, 1955).

IX. EFFICIENCY OF MUSCULAR CONTRACTION

The *efficiency* of a process capable of coupling work to a load is the ratio of the work actually done to the maximum amount that could have been done. The maximum amount of work that can be derived from a process depends, of course, on the mechanism of energy conversion. In a mechanical process, a given potential energy is made available for conversion into work. The fraction of this energy not appearing as work is manifested as heat. Thus the maximum work a mechanical system can do is $W - Q$, and the *mechanical efficiency* is

$$E_{\text{mech}} = \frac{W}{W - Q} \tag{57}$$

Clearly, since a mechanical process is characterized by the fact that $Q \leq 0$, that is, the flow of heat is always from the system to the environment, its mechanical efficiency can never exceed unity.

Consider next a mechanochemical system in which work is derived from a chemical reaction proceeding to extent ξ. According to the second law of thermodynamics, the maximum work that can be derived from an isothermal, isobaric reaction is $-\Delta F = -\xi \Delta\tilde{F}$. Therefore the *thermodynamic efficiency* is defined as

$$E_{\text{thermo}} = \frac{w}{-\xi \Delta\tilde{F}} \tag{58}$$

and sliding contraction mechanisms cannot be differentiated simply on the basis of heat and work measurements on muscle contracting at steady speeds.

The difference between the mechanical and the thermodynamic efficiency is discussed. The mechanical efficiency can be evaluated from measurements of only heat and work. For calculation of the thermodynamic efficiency, however, the free energy change for the net chemical reaction, at the concentrations of the components *in situ*, as well as the work, must be known. This distinction is important since the theoretically meaningful function for a mechanochemical process like muscular contraction is the thermodynamic, and not the mechanical, efficiency.

References

For more complete treatments of thermodynamic theory, the reader is referred to one of the many excellent treatises on the subject. The fundamentals are clearly explained in the text by Zemansky (1951). A more elegant exposition can be found in the treatise of Guggenheim (1957).

The thermodynamics of muscle contraction has been discussed several times by Hill (1951, 1956). Analyses have also been made by Ramsey (1944), Pryor (1950), Buchthal, Kaiser, and Rosenfalck (1951). Polissar (1952), Szent-Györgyi (1953), and Huxley (1957).

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The mechanical efficiency of frog sartorius muscle can be calculated from Eqs. (13) and (14) by writing Eq. (60) as

$$E_{mech} = \frac{\dot{w}}{\dot{w} - \dot{Q}} \tag{60'}$$

Since both w and $\dot{Q}$ are functions of the contraction velocity, $E_{mech} = E_{mech}(v)$. The thermodynamic efficiency will vary with the contraction velocity in the same way, differing from $E_{mech}(v)$ only by the factor $\widetilde{\Delta H}/\widetilde{\Delta F}$ (Eq. 63). The two efficiency functions will not be equal unless $\widetilde{\Delta H} = \widetilde{\Delta F}$ at the *in situ* concentrations of the chemical species involved in the driving reaction.[10]

X. CONCLUSIONS

For the analysis of muscular contraction as a mechanochemical process, the contraction cycle is divided into two phases. The first is a relatively rapid process in which the driving reaction A proceeds to some extent and energy leaves the system as work and heat. This is followed by a slower recovery process in which reaction A is reversed through chemical coupling with another reaction, R. The relation between the energy fluxes in these two phases suggests that a single parameter, the extent of reaction, can be used to describe all the chemical changes in the initial phase, regardless of the particular mechanical response of the muscle.

The first law of thermodynamics, applied to the experimental relations of Hill for the rates of work and heat production, shows that in muscle the rate of the driving reaction A is controlled by the speed of shortening. This can be explained if the driving reaction is coupled to a geometrically constrained reaction. In particular, if the sites for the latter reaction are distributed on complementary sets of filaments which move relative to each other as the muscle shortens, the rate of reaction will depend on the velocity of shortening. The relation between the chemical reaction rate, $\dot{\xi}$, and the mechanical contraction rate, v, is worked out for both sliding and folding kinematics of shortening. The kinetics of *both* mechanisms can be adjusted to fit the hyperbolic relation between $\dot{\xi}$ and v derived from the measurements of Hill. Thus, unless the interacting sites can be independently characterized, the folding

[10] The reader is referred to section VIII for a discussion of the concentration dependence of ΔF. The value of ΔH is independent of concentration for ideal solutions.

CHAPTER VIII

Comparative Physiology of Activation of Muscles, with Particular Attention to Smooth Muscles

C. LADD PROSSER

I. INTRODUCTION

The comparative viewpoint in muscle biology gives a perspective which emphasizes the inadequacies of the traditional laboratory study of only a few muscles of frog and mammals. Our knowledge of contractile proteins is based largely on actomyosin extracted from the rabbit psoas; many students of physiology base their ideas of muscle function solely on the frog gastrocnemius; and histologists commonly teach that there are three types of muscle—striated, cardiac, and smooth.

Actually, when one looks at a variety of animals, or even at different muscles in the same animal, one notes a very wide spectrum with respect to speeds, holding power, dependence on facilitation, activation by nerves, and histology. Except for some visceral types of muscle, and myogenic hearts, activation is normally via motor nerves, and many of the differences among muscles are referable to their innervation. From the fastest muscles (such as the indirect flight muscles of insects and the ocular muscles of mammals) to the slowest muscles (body wall muscles of sea anemones and vertebrate visceral muscles) muscles cover a span in speeds of some ten thousandfold. Fast phasic muscles relax within a few milliseconds, while some molluscan muscles can remain contracted for minutes. Fast postural muscles of vertebrates give a

conduction can be independent of nerves. In mammals such non-striated muscles as the nictitating membrane and pilomotors are more like postural than visceral muscles in nervous control. There are, therefore, many kinds of nonstriated muscle, and it is here proposed that the term smooth muscle be restricted to vertebrate visceral muscle.

Nonstriated muscles are not necessarily primitive; many coelenterate muscle fibers are striated, and vertebrate smooth muscle may, in fact, be a recent specialization. In conclusion, a wide range of histological types of muscle exists, not just the three types of elementary histology. The elucidation of functional correlations with histological patterns remains to be done.

III. ALL-OR-NONE CONTRACTIONS

The principle of classical muscle biology that single fibers contract maximally, once they are excited, is of limited applicability; there are more kinds of muscle in which contractions are graded than in which the all-or-none law applies. The all-or-none concept, first enunciated for cardiac muscle, was extended to individual fibers of skeletal muscle by Keith Lucas in 1905. Stimulating by small electrodes and recording the contractions of single fibers or groups of known numbers indicated that many skeletal fibers contract maximally, if at all (Pratt and Eisenberger, 1919). The action potentials from single motor units (groups of muscle fibers innervated by one motoneuron) are not graded, and potentials recorded by intracellular microelectrodes from various postural muscles show all-or-none conduction within single fibers. However, in striated fibers under conditions where muscle conduction does not occur, as in stimulation with electrodes a few micra in diameter (Gelfan and Bishop, 1932) or with large transverse electrodes (Sichel and Prosser, 1942) one can observe graded contractions which show no refractory period and no conducted action potential. The all-or-none nature of the twitch is a consequence of conduction in a membrane with a refractory period and is not inherent in the contraction mechanism. It is also not inherent in the activation by the nerve impulse at the motor end-plate, even though the motor impulse is all-or-none. End-plate potentials are graded and, as will be shown below, the many muscles which are activated by the end-plate potential and not by a conducted muscle impulse all show graded contractions.

Vertebrate hearts, under appropriate conditions, contract in an all-or-none fashion and cannot be tetanized. By contrast, heart muscles of

maximal twitch to a single stimulus, whereas some crustacean and coelenterate muscles require a train of impulses for a detectable contraction. In general, high speed is often correlated with striations, yet many nonstriated muscles of invertebrates are faster than some striated muscles of vertebrates. There is virtually no evidence for a correlation of speeds of contraction and relaxation with differences in muscle energetics or in muscle proteins, although such correlations deserve much more consideration. It is most remarkable that similar contractile machinery should provide such a range of speeds and tension maintenance.

II. THE SPECTRUM OF HISTOLOGICAL TYPES

Muscle histology is considered in other chapters of this volume and this chapter on comparative physiology can only point out the inadequacy of the usual histological classification. Differences in speed and mechanical properties may be correlated with but not explained by histological properties. High speed is often found in muscles with close striations, long fibers, and small amounts of connective tissue. The indirect flight muscles of insects have very large fibrils (sarcostyles) and very narrow striations; the slower insect leg muscles have more and thinner fibrils (Edwards *et al.*, 1956). Vertebrate white muscles have many closely packed fibrils and peripheral nuclei, while red muscles have relatively more sarcoplasm and some central nuclei. Except in cardiac muscle, vertebrate striated fibers are not branched, but some insect striated muscle fibers are branched. A few muscle fibers (heart of the tunicate *Ciona*) are said to be striated on one side and nonstriated on the other (Bozler, 1927).

Some muscles which were formerly thought to have spiral striations have recently been shown to have a helix of peripheral fibrils which give the appearance of striations; this is well shown in earthworm body wall muscles (Hanson, 1957; Hanson and Lowy, 1957). Visceral nonstriated muscles of vertebrates have fiber lengths of 0.1–0.5 mm.; in the long muscles of bivalve molluscs, nonstriated fibers may be 1 to 2 cm. long. The nonstriated fibers in some holothurian muscles are profusely branched. The short-fibered, nonstriated muscles of some invertebrates, e.g. the proboscis retractors of Phascolosoma, are relatively fast muscles, yet the fibers resemble those of vertebrate smooth muscle in dimensions. It appears that in all invertebrate short-fibered, nonstriated muscles, conduction is by intrinsic nerves, whereas in vertebrate visceral muscle,

quency of stimulation. The first general cause is neuromuscular facilitation. When not all the motor endings are stimulated, that is, when the intensity of stimulation is submaximal, neuromuscular facilitation results in the activation of more fibers upon repeated stimulation. This effect with submaximal stimuli is especially important in noniterative muscles. When a normal frog sartorius is stimulated maximally, the tetanus/twitch ratio is about 1.8 at 15°C., and when stimulated submaximally the percentage increase in a tetanus is greater and the tetanus/twitch ratio may be 30% greater. After curarization or after degeneration of the motor nerve, the tetanus/twitch ratio is the same for maximal as for submaximal stimuli. In iterative muscles, especially where contractions are not all-or-none, neuromuscular facilitation is a requirement; this will be discussed later in some detail for various types of muscle activation.

A second important factor responsible for increased tension with increasing frequency of stimulation, and the only factor in noniterative muscles stimulated maximally, lies in the mechanical properties of the muscles. A variety of evidence on mechanical properties indicates that the contractile elements of muscle have some elastic elements in series (e.g. tendons) and other elastic elements in parallel (e.g. sarcolemma and interfiber connective tissue). According to Hill's theory of activation (Hill, 1949, 1951), the active state builds up very rapidly during the latent period and starts to decline well before the peak of contraction is reached. The initial part of a twitch contraction takes up slack in the series elastic elements and by the time maximum contraction is reached, the active state has already declined. When stimulated repetitively, all energy after the first few contractions can go into development of tension. The tension becomes maximal at the frequency at which mechanical fusion is truly complete.

This mechanical basis for frequency-dependence is important for all muscles; it has been most studied for noniterative vertebrate skeletal muscles. In a frog sartorius, for example, any agent such as nitrate which prolongs the active state reduces the tetanus/twitch ratio (Ritchie, 1954). As the muscle is cooled below an optimal temperature, the twitch tension increases while the tetanic tension is unchanged or slightly reduced. At supraoptimal temperatures, tetanic tension declines and twitches increase slightly. In the cold, the rates of contraction and relaxation are slowed, twitch tension rises, and frequency of mechanical fusion is lowered (Table I, from Nielsen and Prosser, unpublished

molluscs and crustaceans, although the fibers are often branched, give graded contractions and can be smoothly tetanized.

In those muscles which have all-or-none contractions because of propagated muscle impulses, gradation of movement is largely by variation in number of active motor units. In the many muscles in which fiber contraction is not all-or-none, gradation of movement is largely by variation in frequency of activation from the central nervous system.

IV. FREQUENCY-DEPENDENCE OF TENSION

A wide variation exists among muscles in their dependence on frequency of stimulation. Those muscles in which a single stimulus elicits a good contraction and in which a tetanic contraction is rarely more than two or three times a twitch, that is, muscles which show little frequency-dependence, are said to be noniterative. In a very few muscles, there is little or no increase in tension with increasing frequency of nerve activation; this independence of frequency is found in squid mantle (Prosser and Young, 1937) and in the body wall of the polychaete *Branchiomma* (Nicol, 1952) where the motor innervation is by giant axons; small motor axons elicit localized contractions of the same muscles. Most postural muscles, however, show some increase in tension on repetitive stimulation, even when the applied stimuli are of maximal intensity. There may be simple summation, or addition of tension, when a second contraction occurs before the first has subsided. Tension increases up to some maximal frequency and tetanus/twitch ratios between 1.5 and 3.0 are common. Examples of such summation are found among the fast skeletal muscles of vertebrates. Most noniterative muscles give all-or-none type contractions.

Iterative muscles are those in which there is a marked dependence on frequency of stimulation. These muscles show true facilitation, in which contractile tension increases disproportionately on repetitive stimulation. Frequently, iterative muscles are incapable of a twitch response to a single motor impulse. The increase in tension upon going from a low to a maximum frequency may be manyfold. Such iterative responses have been best studied in crustacean and coelenterate muscles, less well in the "slow" motor system of amphibians. Some crustacean leg muscles are noniterative when stimulated via a "fast" motor axon and iterative by a "slow" excitor axon.

Several factors contribute to increased tension with increasing fre-

muscles, and to very slight degree in noniterative, gradation is by variation in frequency of stimulation. A third method of gradation is found where multi-neuronal innervation occurs, as in crustaceans and insects; different motor axons can elicit fast or slow contractions and responses of varying frequency dependence. In addition, in crustaceans, inhibitory axons add a further refinement in gradation of movement.

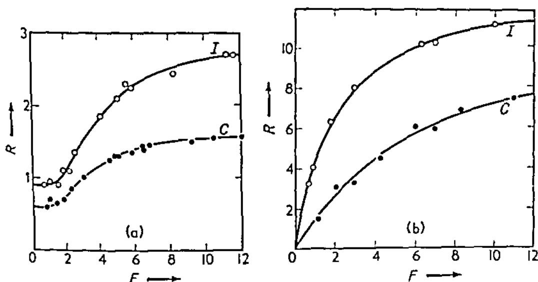


FIG. 1. A. Isometric contraction of cat soleus on maximal stimulation of motor nerve at varying frequencies. I, *before*, and C, *after* cutting some of the nerve fibers; R, responses; F, frequency. From Rosenblueth and D.M. Rioch (1933), p. 366. B. Isotonic contraction of nictitating membrane of cat in response to maximal stimulation of cervical sympathetic at varying frequencies (F), when nerve was intact (I), and after it was partly cut (C).

V. PATTERNS OF NEUROMUSCULAR ACTIVATION

Muscles are normally activated to contraction by impulses in motor nerves. Myogenic hearts and a few visceral muscles are capable of spontaneous contraction and in these there is conduction from muscle fiber to fiber and regulation by extrinsic nerves. The first step, then, in initiation of most muscular activity is nervous excitation. Second, in postural muscles there must be conduction either along the muscle fiber itself or in parallel nerve fibers which activate the muscle at numerous points of entry. Third, the excitation must, in some manner, be transmitted from the surface of the fiber to the contractile elements. Finally, the chemical machinery of the contractile proteins passes through its complex chain of events in contraction and relaxation. While the basic principles in the sequence of neuromuscular excitation, conduction,

TABLE I

CONTRACTION DATA OF FROG SARTORIUS MUSCLES AT DIFFERENT TEMPERATURES

Temperature (°C.)	Contraction time (msec.)	Time for 1/2 relaxation (msec.)	Optimum frequency per second	Twitch tension (g.)	Maximum tetanus tension (g.)	Tetanus: twitch (ratio)
11.6	110±15.4	230±31.9	12.6±2	11.4±1.2	16.7±1.6	1.76
16.4	75± 7.6	110± 9.7	16.4±1	10.2±1.1	18.6±1.8	1.99
21.9	40± 5.3	50± 4.7	25.1±1.6	7.5±0.9	23.4±1.5	2.38
25.2	23± 1.6	33± 3.4	31.3±2.5	10.5±1.3	20.1±2.0	2.08

data). In general, those slow muscles with long contraction and relaxation times have low fusion frequencies, often less than 5 per second. In some of these, e.g. the body wall retractors of holothurians, there is a relatively large amount of interfiber connective tissue. However, there is no information regarding the duration of the active state in such muscles. In others, as in the indirect flight muscles of insects, the frequency of contraction is dependent on mechanical inertia and may greatly exceed the frequency of fusion when stimulated via the motor nerve. A comparison of tension-frequency curves of different muscles with their mechanical properties and active state duration would be very useful.

A third cause for increased tension with repetition has not been much considered lately; this is the possibility that some activating agent, such as a chemical mediator or a product of the mediator action, can pass from muscle fiber to fiber beyond the activated nerve endings. This was suggested by Rosenblueth's observation (Rosenblueth and McK. Rioch, 1933) that stimulation of a fraction (e.g. one-half) of motor axons to a postural muscle of a mammal results at all frequencies in that same fraction of the tension due to stimulation of all the axons in the nerve, whereas when a fraction of the autonomic axons activating some smooth muscles is stimulated, the tension rises on repetition to approach the tension obtained from stimulating all the autonomic axons (Fig. 1). In visceral smooth muscle, conduction can be independent of nerve fibers and excitation can spread from muscle fiber to fiber.

Gradation of movement in noniterative muscles has been indicated as due largely to variation in number of motor units. In iterative

and the motor nerve is stimulated, all-or-none conducted muscle spikes are recorded until the active electrode approaches the region of innervation; here a foot on the action potential appears. Under curarization, a graded potential of slower rates of rise and fall than the muscle spike is recorded in the region of the end-plate. When curarization is incomplete, muscle spikes are seen to emerge from the larger end-plate potentials (e.p.p.'s) (junction or postsynaptic potentials, p.s.p.'s) (Fig. 2) (Eccles *et al.*, 1941). By intracellular recording, the e.p.p. is shown to represent only partial depolarization (less than one-fourth of spike height in frog), to have a rate of rise about one-third that of the spike, to last longer (Table II) (Fatt and Katz, 1951; Kuffler, 1952a), to show no refractoriness, but to be capable of summation with closely spaced nerve impulses. The conducted spike overshoots zero potential, that is the membrane reverses its sign, whereas the e.p.p. is graded and never

TABLE II

COMPARISON OF SPIKE AND END-PLATE POTENTIAL FOR FROG SARTORIUS[a]

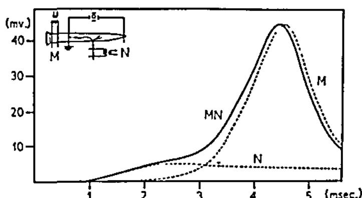
Condition	Spike	End-plate Potential
Initiated by	outward local currents	acetylcholine
Conduction	all-or-none, about 1 m./sec.	local; electrotonic spread
Action potential (resting potential 95 mv.)	130 mv.	30–50 mv. (depends on conditions)
Rise time (20 to 100% peak)	0.45 msec.	1.5–2.1 msec. (at about 10°C.)
Fall time (peak to 50%)	2 msec.	3.8 msec (at 10°C.)
Conductance rise phase	specific increase Na permeability	increased permeability to all ions
Conductance fall phase	specific increase K permeability	
Sensitivity to resting potential and Na replacement	independent of membrane potential, sensitive to Na concentration	proportional to membrane potential, relatively insensitive to Na level
Rate of rise of spike	at distance 670 v. per second	in end-plate region 220 v. per second

[a] Resistance of membrane nearly same in all regions, 4000 ohm cm^2.; capacity of membrane similar in all regions, 6–8 μ cm^{-2}, space constant of membrane relatively uniform, 2–2.4 mm.

Data from Fatt and Katz (1951; Katz (1956); Nastuk and Hodgkin (1950); Nastuk (1953).

activation of the chemical system, contraction, and relaxation may be roughly similar in all muscles, nature has used a variety of modifications in different kinds of muscle.

The most striking variations in excitation and conduction lie between those long-fibered muscles (whether striated or not) where an impulse at a single nerve ending sets off a conduction wave of excitation down the muscle fiber, and those where the nerve impulse causes only local effects and conduction is in nerve axons to the multiple endings on one



[illegible] stimulation of muscle; full line, potential due to combined MN stimulus (e.p.p. plus muscle spike). From Eccles *et al.* (1941), p. 362.

muscle fiber. In short-fibered muscles, conduction may be by nerve fibers or from muscle fiber to fiber. The various patterns of neuromuscular excitation have recently been summarized by Hoyle (1957a); only a brief comparison is, therefore, necessary.

A. The "Fast" Motor System of Vertebrates

The pattern of neuromuscular excitation by fast motor axons in striated muscle of mammals and frogs is best known, although this is not the most common and generalized mode of excitation. The sequence of events is as follows:

motor nerve impulse → liberation of transmitter, acetylcholine → junction (end-plate) potential (e. p. p.) → muscle impulse → activation of contractile system

There are two all-or-none steps, the nerve and muscle impulses; the velocity of the muscle impulse is 1–3 m. per second, that of the nerve impulse is tens of meters per second. As small recording electrodes are moved along the outside of a fiber of a muscle such as a frog sartorius

potential appears to result from a nonselective increase in ion permeability, a short-circuiting of the end-plate region (Katz, 1956). Properties of muscle spike and end-plate potential are compared in Table II (Nastuk and Hodgkin, 1950; Nastuk, 1953).

Under conditions of calcium lack or magnesium excess, the end-plate shows spontaneous miniature e.p.p.'s, relatively similar in size, which may correspond to quantal liberation of acetylcholine (Castillo and Katz, 1955). The end-plate potential is the summation of many mini-

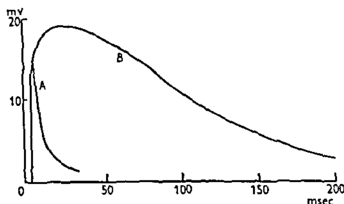


Fig. 4. Superimposed tracings of e.p.p.'s. A, from low-sodium muscle; B, from same muscle after prostigmine bromide (10^{-6}). From Fatt and Katz (1951), p. 338.

ature e.p.p.'s, each corresponding to a quantum of acetylcholine (Lilly, 1956; Burke, 1957). The picture, in summary, is that motor nerve impulses liberate acetylcholine in quanta and that this acts on the sole plate membrane by increasing its ion permeability, that the resulting end-plate potential can be graded, and that at a critical magnitude it starts a muscle impulse which is conducted by the muscle membrane in an all-or-none fashion.

The mode of activation of the contractile elements by the muscle impulse is not known. However, localized stimulation with point electrodes suggests that the signal passes inward at the Z-line in frog muscle and at the boundary of A and I bands in crustacean muscle (Huxley, 1957). Possibly intra-fiber conduction is by the sarcoplasmic reticulum which has been visualized by electron microscopy.

A variety of evidence indicates that the preceding sequence of stimulation of skeletal muscle applies to all postural muscles of mammals and to the phasic muscles of rapid movements in amphibians. Such muscles are innervated by large motor nerve fibers (10–12 μ in diameter and 10–40 m. per second velocity in the frog).

fully depolarizes the membrane. The e.p.p. spreads electrotonically out from the end-plate (Fig. 3) and at a critical amplitude and area elicits the conducted muscle impulse. Local application of small amounts of acetylcholine at the end-plate causes electrical response similar to the e.p.p.; acetylcholine is ineffective on other parts of the muscle fiber membrane. Eserinization greatly prolongs an end-plate potential (Fig. 4). Intracellular records also show that the electric current which crosses the end-plate membrane is only a small fraction

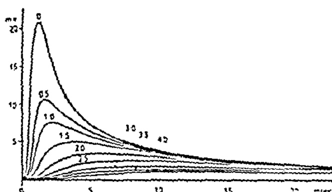


FIG. 3. Tracings of e.p.p.'s at different distances from end-plate focus of frog sartorius. Stimulus artifact served as common point for superimposing records. Numbers give distance in 0.97 mm. from end-plate center. From Fatt and Katz (1951), p. 330

of that necessary to excite the muscle (Kuffler, 1949). When a muscle fiber is stimulated directly so that an impulse is conducted antidromically toward the end-plate, such an impulse fails to leave the end-plate refractory to a nerve impulse arriving immediately after the muscle impulse, hence the e.p.p. involves different membrane components from the muscle impulse (Kuffler, 1952b). The end-plate membrane is not electrically excitable but is normally depolarized by liberated acetylcholine. There is much acetylcholinesterase in the end-plate, particularly along the folds of the muscle beneath the sole plate (Couteaux, 1955). The muscle membrane resembles that of a nerve fiber in becoming selectively permeable to sodium during the rising phase of an impulse. The end-plate, by contrast, is less sensitive to sodium and can produce an e.p.p in response to acetylcholine if depolarized in K_2SO_4 in the absence of Na^+ and when the membrane is "set" by a transverse current. In transmembrane polarization, the end-plate potential, unlike a propagated spike, is proportional in amplitude to the transmembrane potential (Fatt and Katz, 1951). The end-plate

times slower than of the "fast" muscles. The fibers of the slow muscles are stimulated by applied acetylcholine in all regions, and in dilute acetylcholine they maintain a contraction in contrast to the local stimulation and brief response of fast muscles. *Such muscles as the rectus* abdominis are, therefore, useful for assay of acetylcholine. The slow muscles maintain a contraction in response to direct current, the fast muscles respond only on make and break. Innervation is at many endings scattered along the membrane of the slow muscle fiber and activation is by the summation of the many local end-plate potentials (Kuffler and Vaughan Williams, 1953). Conduction is in nerve fibers, *not in the muscle fiber, hence the muscles do not obey the all-or-none* law. Such muscles are ideally suited for slow, graded, and maintained contractions.

Mammalian postural muscles are of the "fast" type. However, in muscle spindles, the sense organs in mammalian muscles, there are intrafusal fibers which are innervated much like the slow muscle fibers of the frog and in which the only action potentials are graded e.p.p.'s (Kuffler *et al.*, 1951). *Sensory impulses from the muscle spindles are* continuous while the muscle exerts a stretch on the spindle, and contraction of the intrafusal fibers interrupts and modulates the sensory discharge. Thus, the type of nervous activation used for tonic contractions in a frog is used for regulating the myotatic reflexes of mammals. The intrafusal fibers of amphibian muscle spindles, unlike those of mammals, are innervated by both "fast" and "slow" axons (Eyzaguirre, 1957).

C. Crustacean Neuromuscular Transmission

Some amphibian and mammalian muscle fibers receive innervation from two (or more) "fast" nerve fibers, but there is no evidence that one muscle fiber receives both fast and slow innervation. In crustaceans, however, multineuronal innervation of fibers is the rule and an entire muscle may receive only a few motor axons, each of which branches extensively. Usually one of the nerve fibers serving all the muscle fibers is an inhibitor; there may be from one to four excitor nerve fibers eliciting contractions of different speeds. The motor unit concept cannot be applied to such muscles. Many motor nerve fibers branch, particularly in appendages, to serve several muscles and the same axon may have different effects on two different muscles (Wiersma, 1941, 1953). The pattern of innervation is very different for differ-

B. The "Slow" Motor System

A frog has other muscle fibers innervated by small motor fibers 5–8 μ diameter and 2–5 m. per second velocity). Such muscle fibers contract slowly and show maintained tonic contractions in contrast to the fast phasic muscles (Tasaki and Mizutani, 1944). Some muscles, e.g. the rectus abdominis, consist entirely of these "slow" fibers, others like the gastrocnemius, are mixed, while others, such as sartorius, are entirely "fast." *The action potentials of muscle fibers innervated by slow motor* fibers are more prolonged than spikes, are graded, and facilitate to give an electrical record resembling a mechanical tetanus (Figs. 5, 6).

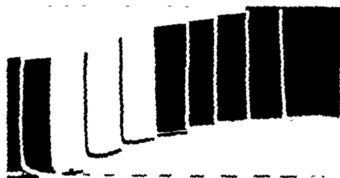


FIG. 5. Twitch fiber and slow fiber contractions in frog iliofibularis. Large motor nerve fibers were stimulated at 1 per second, causing twitches. After the first two twitches, small nerve fibers in the same root were stimulated at 30 per second. Note differences in time course of the two types of contraction, and summation of twitch with slow fiber tensions. From Kuffler and Vaughan Williams (1953), p. 318.



FIG 6. Intra... (a), singl... single fib... p. 310.

The action potential of the whole muscle is like an e.p.p. and the small junction potential decays with a terminal hyperpolarization (Burke and Ginsborg, 1956). Nerve tetanus can depolarize by nearly 50% without a spike response. *Relaxation of the "slow" muscles is some 50 to 100*

agate in the muscle fiber. However, since propagation in the muscle is only one-tenth as fast as in the nerve fibers, since the muscle is profusely supplied with nerve endings, and since most of the muscle action depends on "slow" potentials, muscle propagation plays no essential part in muscle control (Hoyle, 1957a). The different "slow" excitors to a muscle receiving several of them vary in facilitation, speed of elicited contraction, and degree of depolarization. Gradation of movement depends in part on which excitor is made active by the central nervous system.

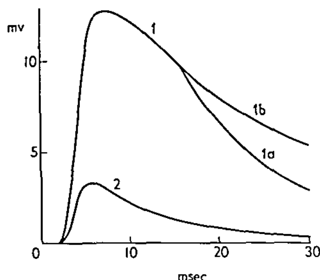


Fig. 8. Superimposed records of electrical responses of opener muscle of hermit crab *Eupagurus*. 1b, phasic e.p.p. type response; 1a, inhibitor stimulated 15 msec. after excitor, accelerates decay of e.p.p.; 2, inhibitor precedes excitor by 1.6 msec., attenuating amplitude of e.p.p. From Fatt and Katz (1953b), p. 378, Fig. 4.

An inhibitory axon may reduce a contraction either with or without simultaneous reduction in the junction potential (Fig. 8). The time course of the reduction of electrical response is longer than that of mechanical inhibition, that is, the inhibitory impulse may arrive some milliseconds before the excitatory and the inhibition effect may persist into the contraction period (Marmont and Wiersma, 1938). When stimulated alone, the inhibitor may elicit no electrical response, or, if the resting potential is low, the inhibitor may hyperpolarize; if the resting potential is increased by anodal polarization, the inhibitor may depolarize, that is, it would tend to restore the membrane toward its normal rest level (Fig. 9) (Fatt and Katz, 1953b).

The small and reverse electrical effects of the inhibitor, the mechanical inhibition without accompanying electrical changes, and the fact

ent muscles and for corresponding muscles of different species (Wiersma and Ripley, 1952). The so-called "fast" excitor fibers evoke quick twitches which are nearly maximum with single impulses. The "slow" excitor fibers elicit little or no contraction with single impulses but by facilitation the contraction builds up at rates varying with frequency and with the particular fiber.

Action potentials from crustacean muscle fibers vary in size much as do the contractions, that is, they facilitate and vary according to which

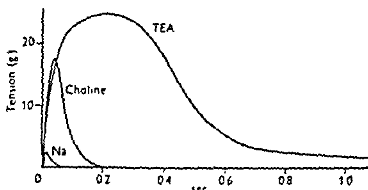


FIG. 7. Superimposed drawings of contractions of flexor of dactylopodite of crab Portunus when leg was perfused with normal Na saline, and when choline and tetraethylammonium (TEA) was substituted for Na. From Fatt and Katz (1953a), p. 191 Fig. 10.

motor axon is active. Microelectrode records from single crustacean muscle fibers show that the potentials in response to each type of axon are graded and are of the e.p.p. type (Fatt and Katz, 1953a). Stimulation of "fast" axons elicits large potentials which facilitate very little; these responses are sometimes compound and on facilitation sum to give a spike-like potential. The depolarization resulting from stimulation of a slow motor axon may be very small, only a few (1–4) millivolts when the resting potential is 70 mv.; even with facilitation the total depolarization is slight (Hoyle and Wiersma, unpublished). The muscle potentials (and contractions, Fig. 7) are markedly prolonged when sodium is replaced by choline and, like frog e.p.p.'s, they are proportional to the magnitude of the membrane resting potential (Fatt and Katz, 1953a). When many muscle fibers in a muscle are sampled by microelectrodes, it is found that some are innervated by "fast" axons, some by "slow", and many by both types (Wiersma, 1957; Furshpan, 1953). The "spikes" sometimes elicited by fast axons and also those initiated by direct transmembrane stimulation may prop-

muscle elements contained in a very small space. Several patterns of nervous activation have been described by Hoyle. In general, most insect muscles, particularly leg extensors and flexors, receive two excitor nerve fibers. In some muscles (e.g. levator tibiae of *Locusta*), all fibers receive branches from each of the two axons; other muscles (e.g. flexor tibiae of *Locusta*) may be divided into several motor units, each receiving two axons (Hoyle, 1955a). A single end-plate may receive branches from each of two axons and end-plates often occur at intervals of 40 to 80 μ along a muscle fiber.

Stimulation of the "fast" axons elicits twitch-like contractions which do not facilitate, whereas stimulation of the "slow" axon gives graded facilitating contractions.

Intracellular recording reveals that the fast fibers evoke e.p.p. type responses which facilitate and may represent more than two-thirds depolarization (resting potential = 60 mv.). In addition, spike-like responses which often overshoot zero occur after the e.p.p.; spikes disappear at low temperatures or in high Mg^{++} or low Ca^{++}. Direct transmembrane stimulation can also produce spikes. However, the size of the spike response varies in different parts of the muscle fiber; the spikes do not propagate but are localized (Hoyle, 1955b).

Stimulation of "slow" axons results in several types of response, typical small e.p.p.'s which may facilitate as much as sixfold, others which are large, may be compound, and may give depolarization nearly as great as a "fast" response. Also, slow fibers elicit in some muscles a slow depolarization which does not facilitate but does summate to a plateau of low depolarization. No true inhibitors have been identified, but Hoyle (1955b) has found in the metathoracic leg of a locust a third axon which can hyperpolarize when the resting potential is low; its function in muscle coordination is not known. In summary, insect leg muscles are activated by fast and slow motor axons, and there is much facilitation; electrical responses, while large and of both junctional and spike types, are local; conduction is in the motor nerve fibers. The insect leg muscle fibers can be electrically excited to give spike-like potentials which do not propagate. Electrical records from grasshoppers free to move about in a cage show that normally most activity is brought about by the slow fibers but that occasional reinforcement by a burst of "fast" impulses occurs (Hoyle, 1957b).

that electrical inhibitions may have a longer effective period than mechanical inhibition make it unlikely that inhibition *needs* to include an electrical step at the muscle membrane. Similarly, some of the slow excitors elicit good contractions with depolarization of only one-seventieth of the resting potential (Hoyle and Wiersma, 1958). Hence it has been proposed that in crustacean muscle, some nerve impulses may act directly on the mechanism of activation of the contractile elements. A whole muscle may function as a single motor unit, but it may give

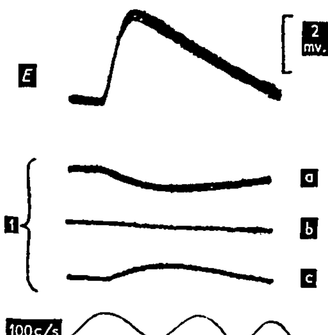


FIG. 9. Intracellular potentials in opener of claw of hermit crab. *E*, e.p.p. due to stimulation of motor axon, I, electrical potential changes due to stimulation of inhibitor. The resting potential in Ib and E was 73 mv. In Ia, it was lowered by cathodal polarization to 48 mv., and in Ic it was raised to 95 mv. Note dependence of I response on level of membrane potential. From Fatt and Katz (1953b), p 382.

contractions of different speeds and magnitudes according to which excitor is active, the frequency of its firing, and the possible simultaneous activation of an inhibitor. Gradation of movement is much more peripheral and more economical of nerve impulses than in vertebrates.

D. MUSCLES CONTROLLING LEG MOVEMENTS IN INSECTS

Insects, like many crustaceans, have the problem of carrying out a variety of coordinated graded movements at high speeds by nerve and

muscle elements contained in a very small space. Several patterns of nervous activation have been described by Hoyle. In general, most insect muscles, particularly leg extensors and flexors, receive two excitor nerve fibers. In some muscles (e.g. levator tibiae of *Locusta*), all fibers receive branches from each of the two axons; other muscles (e.g. flexor tibiae of *Locusta*) may be divided into several motor units, each receiving two axons (Hoyle, 1955a). A single end-plate may receive branches from each of two axons and end-plates often occur at intervals of 40 to 80 μ along a muscle fiber.

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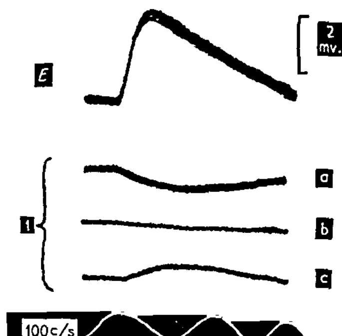


Fig. 9 Intracellular potentials in opener of claw of hermit crab. E, e.p p. due to stimulation of motor axon, I, electrical potential changes due to stimulation of inhibitor The resting potential in Ib and E was 73 mv. In Ia, it was lowered by cathodal polarization to 48 mv., and in Ic it was raised to 95 mv. Note dependence of I response on level of membrane potential. From Fatt and Katz (1953b), p 382.

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E. Resonating Muscles of Insects

In the lower orders of insects, such as Orthoptera, Odonata, and Lepidoptera, the wings are moved directly by muscles which are similar in properties to the leg muscles just described. Wing beat frequency is determined by the nervous discharge, and the muscle can be driven up to a fusion frequency of about 30 to 70 per second by stimulating the motor nerve (Fig. 10) (Roeder, 1951). The normal rate of beat is not

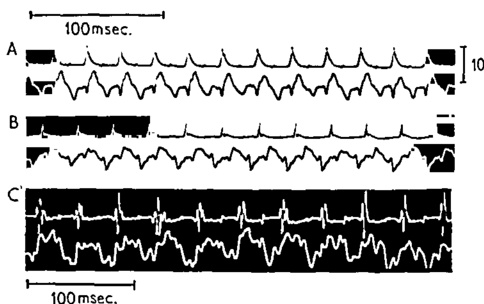


Fig. 10. Movements of thorax (upper traces) and spike potentials (lower traces) during flight. A, in moth *Agrotis* with wings; B, without wings; C, *Periplaneta* with wings. Spikes and thoracic movements synchronized. From Roeder, (1951).

altered by cutting away the wing to reduce inertia. In the flies and hymenopterans, however, the wings are moved by indirect flight muscles which are attached by a complex skeletal lever system. In these insects, wing beat and haltere frequencies above 500 per second are frequently found and the beat is sensitive to the inertia of the load (Pringle, 1949). When the wing of a midge was clipped, a beat frequency of 2218 per second was recorded, this means that a complete contraction and relaxation must occur in 0.45 msec. (Sotavolta, 1953). Similarly, the muscles which move the resonating sound-producing membranes, or tymbal, in cicadas may in some species beat at frequen-

cies of about 1000 per second and are then sensitive to the inertia of the tymbal (Pringle, 1954). In both the fast indirect flight muscles and fast tymbal muscles, there may be several contractions for each motor nerve; no direct correlation may exist between nerve frequency and muscle beat (Fig. 11) (Roeder, 1951, Hagiwara, 1953). The muscle may contract more frequently than it can be driven by nerve stimulation. The explanation seems to be that both types of these fast muscles are attached to skeletal elements which, when pulled slightly by the

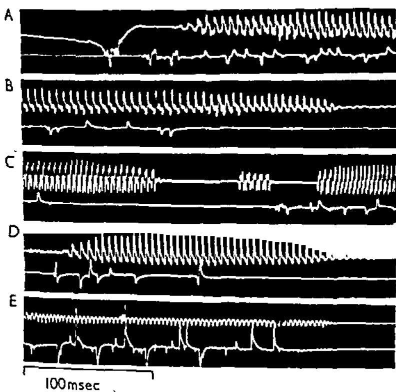


FIG. 11. Action potentials (lower traces) and thoracic movements (upper traces) during flight in several flies. A, onset of flight, *Calliphora*; B, termination of flight, *Calliphora*; C, irregular flight after amputation of wings, *Calliphora*; D, short mid-air flight movement, *Eristalis*; E, termination of flight in *Lucilia* after wing amputation. Lack of synchrony between thoracic movements and potentials. From Roeder, (1951).

muscle, snap inward and thus shorten the muscle slightly (Pringle, 1957a), On shortening, the tension of the muscle drops to zero, and the skeletal element by its own elasticity (tymbal) or by contraction of an antagonitic muscle (paired indirect flight muscles), then snaps out and thus stretches the muscle which redevelops tension rapidly (Boet-

tiger, 1957; Boettiger and Furshpan, 1952, 1954; Pringle, 1957b). So long as the active state left by one nerve impulse remains, the muscle can lose tension on release and redevelop it on stretch several times. A myogenic rhythm, sensitive to inertial load and more frequent than activating nerve impulses, is responsible for the fast wing beats of flies, bees, and wasps, and for the high sounds of cicadas.

F. Activation of Nonstriated Skeletal Muscles of Invertebrates

Among vertebrates, the fast phasic muscles are usually cross-striated and the slow tonic muscles are usually nonstriated. Yet the contraction rates of some invertebrate nonstriated fibers, particularly in sipunculids, echinoderms, and molluscs may be as fast as the rate of many striated muscles. Speed may be related more to innervation than to striations.

1. *Long-Fibered Nonstriated Muscles*

Long-fibered, nonstriated muscles occur in molluscs, especially in the adductor muscles of bivalves. These closing muscles of clams and oysters are composed of two parts: a fast, sometimes striated, portion which closes the valves, and a slow, nonstriated part which maintains tension for hours or even days. The byssus retractor of *Mytilus* has been much studied; it has the remarkable property of giving twitch-like (phasic) contraction to brief alternating current (AC) or pulsed direct current (DC) and of remaining contracted for many minutes after a direct current stimulus lasting a few seconds (Winton, 1937; Fletcher, 1937). If, during the tonic contraction after DC stimulation, a brief series of pulses is applied, the muscle promptly relaxes. Two hypotheses have been proposed to account for maintenance of tension in these molluscan muscles. One is that the contraction is tetanic; this is based on the fact that in reflex tonic contraction, some continuous electrical activity is detected in the adductor muscle so long as innervation is intact and also that motor nerve stimulation at frequencies as low as 5 to 15 per hour can maintain contraction (Bayliss *et al*, 1930; Lowy, 1953; Hoyle and Lowy, 1956). Asynchronous "spontaneous" electrical activity in isolated byssus retractor has been attributed to nerve cells by some (Lowy, 1953) and to injury at nerve endings by others (Twarog, personal communication; Johnson, in press). Also, nerve cells in the muscle have been claimed (Lowy, 1955) and denied (Abraham and

Minker, 1957; Twarog, personal communication). It seems probable that tonus in intact animals requires infrequent but continuous nervous stimulation.

The alternative hypothesis of tonus is that in molluscan holding muscles a "catch mechanism" exists. A scallop or oyster can close against a piece of wood and then retain the degree of closure after the wood is removed. If the isolated byssus retractor of *Mytilus* is partly released during tonic contraction, it fails to redevelop tension unless restretched, whereas if similarly released during phasic contraction, tension is redeveloped as in striated muscle. Also, if stimulated with DC when at about 80% of its "rest" length, the muscle may become stiff as shown by resistance to stretch, without developing tension (Johnson, in press). Short (60 msec.) pulses at 1 sec. intervals elicit small contractions but keep the muscle in such a state that if stretched, it develops considerable tension. The same pulses at 60 msec. intervals give a strong fast contraction which can then be maintained if the frequency drops to 1 per second, a summation of the DC-type contraction (Johnson, in press).

It seems probable that there is truth in both hypotheses of tonic contraction, that the nervous system does normally discharge at low frequency into the muscle, but that tonus is not tetanic in the vertebrate sense. It appears that the mechanical properties of these tonic muscles are such that relaxation may be exceedingly slow, and that stiffness can develop without contractile tension. There is good evidence for inhibitory nerve fibers to the muscles; appropriate stimulation of the visceral ganglion of *Mytilus* relaxes a retractor muscle which is in tonic contraction (Nieuwenhoven, 1947). Probably AC stimulation is more effective than DC on nerves which induce relaxation.

Action potentials of the isolated byssus retractor are decrementally conducted (Schmandt and Sleator, 1955) and there may be tonic contraction without electrical depolarization (Twarog, 1954). In intact muscles, however, conduction is 13–20 cm. per second and action potentials summate or even fuse (Fig. 13c) (Fletcher, 1937; Prosser *et al.*, 1951). It seems probable that normal conduction is by nerves and that such conduction as occurs in the muscle fibers is electrotonic. Acetylcholine causes a contraction of the byssus retractor which persists long after an initial depolarization and repolarization (Twarog, 1954). Serotonin (5-hydroxytryptamine) in low concentration relaxes the muscle and might be the inhibitory mediator (Twarog, 1954), but after such

relaxation, DC contractions can still be elicited, possibly by direct stimulation.

2. *Short-fibered Nonstriated Muscles*

The nonstriated postural muscles of many invertebrates resemble smooth muscle in that the fibers are short spindles. They have been studied most in coelenterates, echinoderms, and sipunculids. In all of these, conduction appears to be by nerve fibers and, despite their

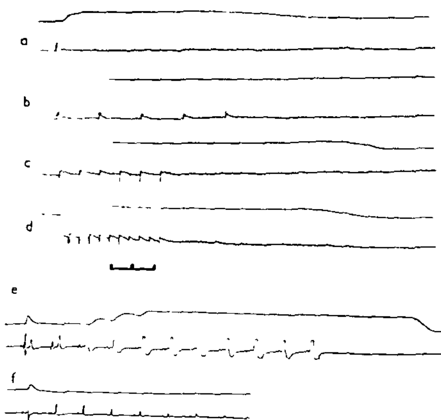


FIG. 12. a–d. Contractions (upper records) and action potentials after shocks (lower records) in longitudinal body wall retractors of the holothurian *Thyone* stimulated at different frequencies Time intervals 1 sec. From Prosser (1954). e–f. Responses of proboscis retractor of *Phascolosoma*, contractions upper line, action potentials lower line, e, both fast (declining) and slow (facilitating) potential responses and both phasic and tonic contractions, f, fast response only.

different histology, the muscles have much similarity to crustacean striated muscles. Some of these remain contracted for many seconds following stimulation (Fig. 12 a–d) and it is possible that they have something comparable to the "catch" mechanism of molluscan muscles.

The proboscis retractors of the holothurian *Cucumaria* show a twitch-like contraction and also a facilitating slow contraction (Pople and Ewer, 1955); the corresponding muscles of *Thyone* give both fast and slow action potentials (Prosser *et al.*, 1951). The contractions of these muscles summate on repetitive stimulation and the contraction is much enhanced by eserinization (Prosser, unpublished data) and is blocked locally by D-tubocurarine (DuBuy, 1936). The longitudinal retractors of the body wall of *Thyone* are activated at close intervals by branches from the radial nerves. Conduction in an excised muscle goes less than a centimeter from a point of stimulation but travels at 17 cm. per second; the action potential is enhanced by eserine (Prosser, 1954).

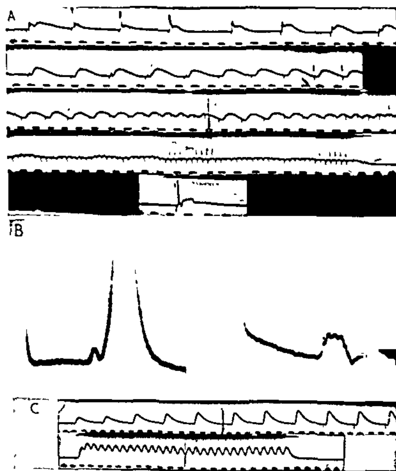


FIG. 13. A, B, responses of proboscis retractor of *Phascolosoma*. A, series of responses showing fatigue of fast and facilitation of slow response; bottom line, both responses to single shock; B, response at high gain before and after local muscle block by D-tubocurarine, C, electrical responses of anterior byssus retractor of muscle of *Mytilus* showing partial fusion of electrical response. From Prosser *et al.*

Holothurian muscles are very sensitive to acetylcholine, and muscles of *Stichopus* have been used for acetylcholine assay (Bacq, 1939). It seems certain that conduction in these echinoderm muscles is by cholinergic nerves.

The proboscis retractors of the sipunculid worm *Phascolosoma* are thin strips of parallel, nonstriated, short fibers, only slightly larger than the fibers of mammalian smooth muscle. These retractor muscles are innervated from the cerebral ganglion at the base of the proboscis. Two types of action potential are found in the proboscis retractors. A fast potential, conducted at 1.3 m. per second, fatigues rapidly on repetitive stimulation and is associated with phasic contractions (Fig. 12e, f). A slow potential, conducted at 0.3 m. per second, facilitates much on repetition and is associated with tonic contractions (Fig. 13 A) (Prosser *et al.*, 1951). The muscle is rich in parallel nerve fibers, some about 2 μ in diameter and others less than 1 μ. After degeneration of the nerve fibers, no conduction occurs, although local contractions can be elicited. Conduction is blocked by procaine and tetracaine; local activation of the muscle is blocked by a drop of D-tubocurarine. Facilitation of the slow potential is enhanced by eserine. Nerve impulses can be recorded just in advance of the muscle responses and corresponding to the two muscle potentials (Fig. 13 B) (Prosser and Melton, 1954). Unlike molluscan muscles, in which fast and slow portions are histologically different, all of the fibers in the *Phascolosoma* muscle appear alike histologically. Microelectrode records show that some of the *Phascolosoma* fibers give only fast responses, some only slow, while many show both fast and slow electrical responses. Neither response represents complete depolarization or overshoot and it seems certain that each is a junction-type potential (Prosser and Sperelakis, unpublished). It is concluded that conduction in the *Phascolosoma* retractor muscle is by intrinsic nerve fibers, that many of the muscle fibers have dual innervation, and that the slow nerve fibers are cholinergic.

Such little evidence as is available for the short-fibered muscles of annelids and coelenterates indicates conduction by nerves. Some coelenterate muscles can give both fast and slow contractions; in the sea anemone *Calliactis*, both types show facilitation; the fast facilitates at intervals up to 2 sec. and the slow to 12 sec. (Ross, 1937). Polyneuronal motor innervation appears to be more common in invertebrate muscles than unineuronal innervation, irrespective of fiber length and the presence or absence of striations. Also, conduction in nerve fibers and lack

of conduction in muscle fibers appears general among various types of invertebrate muscles. Innervation of visceral muscles of invertebrates has not been much studied. The visceral muscles of some insects are striated. In the short-fibered nonstriated muscles of invertebrates, there appears to be no conduction from fiber to fiber such as occurs in vertebrate smooth muscle.

G. Vertebrate Nonstriated Muscles

There are more differences among the various nonstriated muscles of vertebrates than among their striated muscles. In general, vertebrate nonstriated muscles fall into two categories: multi-unit muscles, and visceral or smooth muscles (Bozler, 1948).

1. *Multi-Unit Non-Striated Muscles of Vertebrates*

Muscles such as the nictitating membrane, the pilomotors, and many blood vessels receive extensive innervation and appear to be organized in some type of motor unit plan. Conduction in these muscles is by autonomic nerve fibers. There may be dual innervation by antagonistic nerves (arterioles). Repetitive nerve stimulation results in facilitation of muscle responses. The multi-unit muscles do not show spontaneous activity although they may give rhythmic responses to single shocks or applications of adrenaline (Eccles and Magladery, 1937). The sympathetic innervation is adrenergic, liberating l-nor-epinephrine with variable small amounts of epinephrine. Parasympathetic fibers to such muscles are usually cholinergic. Knowledge of conduction and activation in these multi-unit nonstriated muscles is based almost entirely on early observations, and no studies of these muscles have been made with modern electrical methods.

2. *Visceral (Smooth) Muscle*

Vertebrate visceral smooth muscle differs from other kinds of muscle in many respects. The smooth muscles of various visceral organs have different properties. Smooth muscles show the following in varying degrees: spontaneous rhythmicity, stimulation by stretch, conduction from muscle fiber to fiber, slow contraction and relaxation, and regulation by autonomic nerves and hormones. Differences in contractile proteins and other properties of smooth and striated muscles are considered in other chapters of this book. The slowness of contraction and relaxation result in part from slow excitation processes and in part from

chemical and mechanical differences beyond the scope of this chapter.

a. Rhythmicity. The chemical basis for the rhythmicity of smooth muscle is no better known than for any other pacemaker. Mechanical stretch, acting either directly on the muscle fibers or indirectly through local reflexes, has been suggested as the initiator of rhythmicity. While stretch enhances excitability of smooth muscle structures, spontaneous contractions can often be obtained in the absence of stretch and many chemical agents can alter this spontaneity. In the intact ureter, rhythmic distension initiates waves of contraction (Bozler, 1942a); similarly, distension of a uterus may induce contractions of parturition (Csapo and Goodall, 1954; Csapo, 1955). Both longitudinal and circular muscles of cat intestine freed from ganglionic control contract in response to quick stretch. However, during peristalsis they are normally excited via myenteric reflexes by intraluminal pressure (Evans and Schild 1953a; Bulbring *et al.*, 1958). A plexus-free preparation of cat circular intestinal muscle shows pendular movements (Gasser, 1926) and slow rhythmic local and peristaltic contractions (Gunn and Underhill, 1915), although best peristalsis occurs when the ganglionic plexuses are intact and the myenteric reflexes can function (Klinge, 1951). In normal peristalsis, the initial contraction of longitudinal muscle may result directly from distension, while the second wave or circular contraction follows reflexly (Kosterlitz *et al.*, 1956; Kosterlitz and Robinson, 1957). Peristalsis of the duodenum is reduced by ganglionic blockade with hexamethonium much more in the rabbit than in the cat (Paton and Zaimis, 1949). Microscopic observation of a ring of deganglionated circular duodenal muscle stretched over a glass rod reveals segmenting waves 1.2 mm. wide which pass at about 1.2 cm. per second for a centimeter or more along the length of the tube (Sperelakis and Prosser, in publication); each segmenting wave includes several of the fasciculi of Carey (1921). In taenia coli of the guinea pig, as tension is increased, the resting membrane potential decreases and the frequency of spontaneous spikes increases (Fig. 15) (Bülbring, 1955). Several drugs, for example eserine and dinitrophenol, can dissociate spike frequency from resting membrane potential but the relation of frequency to tension remains; acetylcholine partially depolarizes and increases spike frequency, adrenaline hyperpolarizes and decreases spike frequency (Burnstock, 1958). In general, stretch reduces membrane potential and brings fibers into the zone of firing.

In the ureter, prepotentials have been seen to build up repetitively

at the renal end, prior to the electrical wave of peristalsis (Bozler, 1942a); however, any region of the ureter can, under experimental conditions, initiate spontaneous waves. Intracellular records from intestinal muscle show graded prepotentials (generator potentials) preceding spontaneous spikes (Fig. 14) (Bülbring 1955; Sperelakis and Prosser, in publication). Similar prepotentials are recorded with microelectrodes in the uterus (West and Landa, 1956). These are local generator potentials which may take on the rôle of pacemakers.

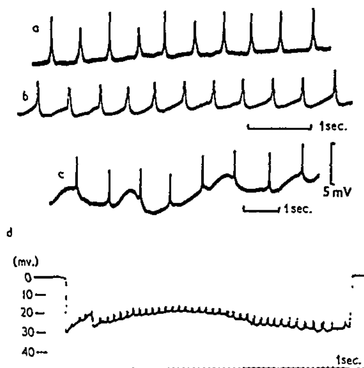


FIG. 14. Intracellular records from taenia coli of guinea pig. Spontaneous discharges showing spikes, some preceded by prepotentials, particularly in b and c. d. Intracellular record of potentials from taenia coli of guinea pig. Penetration indicated by drop of base line. Spontaneous fluctuations in R.P. and spontaneous spikes, some double and some single. From Bulbring p. 306.

A diestrus or castrate uterus is quiet, but one under the influence of estrogen is highly active (Bozler, 1942b; Jung, 1956). Progesterone decreases spontaneity of the uterus. It has been hypothesized that the stimulating effect of estrogen is brought about by an increase in potassium inside the muscle fibers relative to that outside. Analyses of myometrium indicate an increase in intracellular concentration of potassium (Horvath, 1954) although the uterus as a whole may gain relatively more sodium (Kalman, 1957). An increase in the ratio of intracellular

chemical and mechanical differences beyond the scope of this chapter.

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In the ureter, prepotentials have been seen to build up repetitively

a depressant action; as agents for quieting a highly active preparation and leaving it excitable by electric shocks after excitants like barium are washed away, a papavarine-like compound (RO2-4394/2 of Hoffman-LaRoche, Inc.), Serpasil, and tetracaine (Prosser and Sperelakis, 1956), also cooling (Ambache, 1946) are very effective. In the taenia coli, acetylcholine depolarizes slowly and increases the frequency of spontaneous spikes; adrenaline hyperpolarizes and decreases spike durations and frequency (Bülbring, 1957). Cathodal polarization increases spontaneous activity and anodal polarization decreases it (Bülbring, 1957).

Spontaneity in the absence of nerve cells is convincingly demonstrated

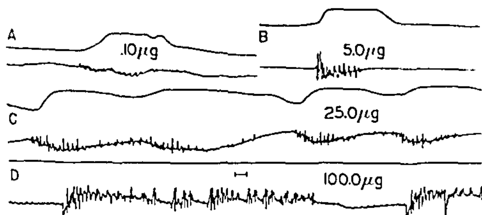


Fig. 16. Spontaneous mechanical (upper records) and electrical (lower records) activity in uterus of castrate rat at various levels of estradiol treatment. A and B, rhythmic contractions and bursts of spikes; C, relaxation incomplete and electrical activity practically continuous; D, continuous low level electrical activity, contraction complete and sustained. Time line 1 sec. From Melton (1956), p. 142.

by the chick amnion in isolation (Pierce, 1933; Ferguson, 1940) or in tissue culture (Lewis and Lewis, 1917). In about the fifth day of incubation, rhythmic contractions begin in the nerve-free amnion and continue to the eleventh or twelfth day (Pierce, 1933; Prosser and Rafferty, 1956). The amnion not only contracts rhythmically but also responds to mechanical or electrical stimuli, is stimulated by barium, acetylcholine, histamine, and is not affected by nerve and ganglion inhibitors like morphine, atropine, and hexamethonium (Evans and Schild, 1953; Kuschinsky *et al.*, 1954).

b. Electrical activity. Some smooth muscles (intestine, uterus) show spike-like action potentials as recorded externally, bursts of these corresponding to contraction waves. In the estrogen-dominated uterus there

to extracellular potassium would be expected to cause an increase in resting potential, whereas the reverse correlation of resting potential with spontaneity was observed in taenia coli (Bülbring, 1955).

Intestinal smooth muscle preparations, from which the ganglionic plexuses have been removed or which have been treated with ganglion-

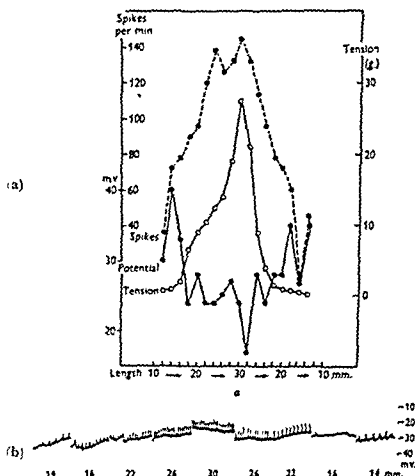


FIG. 15. (a) Effect of progressive stretch and relaxation on membrane resting potential •—•, tension o—o, and rate of spontaneous discharges of spikes •---• in taenia coli of guinea pig. Abscissa, muscle length; (b) records of action potentials in corresponding steps during stretch and relaxation. From Bulbring (1955), p. 206.

blocking drugs, are readily set into enhanced rhythmicity by barium and also by acetylcholine, either of which may establish local pacemakers. Histamine also stimulates the muscle directly. Acetylcholine is normally synthesized by intact intestine, particularly by the mucosa, and maintained tone and movement of villi have been suggested as due to the presence of acetylcholine (Feldberg and Lin, 1950). Adrenaline has

Other smooth muscles, such as ureter (Bozler 1942; Prosser *et al.*, 1955) and stomach (Ichikawa and Bozler, 1955) show prolonged depolarization resembling that of heart muscle. External monophasic records may show depolarization lasting for 2 sec. (Fig. 17). Depolarization at a proximal point on the ureter can be prolonged to such an extent that it outlasts both depolarization and repolarization at a distal point; in such a state a diphasic record shows a negative instead of a positive second phase (Fig. 17 B) (Prosser *et al.*, 1955). The duration of depolarization can be prolonged by a variety of nonspecific agents—

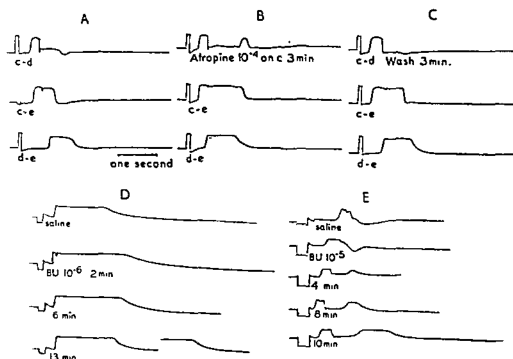


FIG. 17. Action potentials from ureter of rat, electrodes in order a, b, stimulating; c, d, recording, on normal ureter 1 cm. apart, e, on KCl-treated end; c-d, diphasic; c-e and d-e, monophasic records. A, initial responses; B, atropine 10^{-4} applied at c inverted the final diphasic wave and prolonged depolarization; C, partial recovery after saline wash.

D, E, Monophasic and diphasic records from rat ureter, prolongation of depolarization and inversion of diphasic record by tetrabutylammonium 10^{-6} applied at electrode C. From Prosser *et al.* (1955).

high potassium, atropine, higher quaternary ammonium compounds (tetrabutylammonium, tetraethylammonium) (Fig. 17). Acetylcholine prolongs depolarization of the ureter and shortens it in heart muscle. The duration of depolarization is shortened by adrenaline (again unlike heart) and by low calcium in ureter (Fig. 18) (Prosser *et al.*, 1955)

may be rhythmic low-level potentials not associated with movement and then bursts of large spikes at times of contractions (Fig. 16) (Melton, 1956). Intracellular electrodes record lower resting potentials than in striated muscles. Some average values are given in Table III.

TABLE III

ELECTRICAL ACTIVITY IN SMOOTH MUSCLE

Muscle	Resting potential	Action potential	Reference
	(mv.)	(mv.)	
Rat uterus, pregnant	42.3	27.7	West and Landa (1956)
Guinea pig uterus, pregnant	32.6	21.9	Woodbury and McIntyre (1954)
Cat circular intestinal	40	22	Sperelakis and Prosser (in publication)
Guinea pig taenia coli	40	15	Bülbring (1955)
Guinea pig taenia coli	50	60	Holman (1957)

The low resting potentials may be lower than the true values because of damage inflicted by inserting a 0.5 μ electrode into a 3.0 μ muscle fiber. Higher values (50 mv.) were obtained with finer high resistance electrodes (Holman, 1957). However, the ionic gradients in smooth muscle are different from those in striated muscle and the resting potentials are undoubtedly lower. Large doses of oxytocin are said to reverse the polarity of the uterine resting potential in several but not in all species of mammal (Woodbury and McIntyre, 1954).

The action potentials, whether spontaneous or responses to barium or to shocks, are spikes which rarely overshoot zero membrane potential. However, overshoots are recorded with high resistance electrodes (Holman, 1958). Generator potentials and spikes are seen with the sucrose gap electrode much as with intracellular microelectrodes (Burnstock and Straub, 1958). In a single fiber, repeated spikes are relatively constant in size, and they do not facilitate even though they represent incomplete depolarization (Fig. 14). The single fiber action potentials of the taenia coli are relatively insensitive to sodium variation over a wide range (Holman, 1956). Sometimes a fiber gives two spikes with less than 100 msec. between peaks (Fig. 14d) Bulbring, 1955; Sperelakis and Prosser, 1959); yet the refractory period as measured by responses to paired shocks is 1 to 3 sec. The double spikes might represent depolarization of different parts of a cell, or more probably the long refractory period may reside in the interfiber transmission mechanism, and not in the excitability of single cells.

also occur transverse to the long axis of the fibers. The velocity transverse conduction is one-tenth that in the direction of the fib (Prosser and Sperelakis, 1956). Conduction can be blocked by m nonspecific agents, some of which act on nerves or functions—te caine, nicotine, D-tubocurarine—but only at high concentratic The threshold concentrations for block by morphine, nicotine, an number of other drugs is some hundred times lower on intact tl on deganglionated intestine. D-Tubocurarine at 10^{-4} blocks c



FIG. 19. Dual beam records of action potentials from rat ureter at high am fication. Large deflections at right, muscle responses. Small deflections, presp (nerve impulses) Upper beam from active electrode 1 5 cm from that of lo beam. Time 1 sec. From Prosser *et al.* (1955).

duction in a rat ureter (Prosser *et al.*, 1955) whereas in the Pł colosoma proboscis retractor, where conduction is by nerves, tu curarine blocks only the local response and leaves through conduct (Prosser and Melton, 1954). The velocity in nerve-free amnion a plexus-free intestinal muscle is similar to that in ureter and uterus w nerves present. In the ureter, one can record nerve impulses in au

and stomach (Bozler, 1942b). Why some smooth muscles give flat-topped action potentials and others give spikes is unknown. It is of interest that repolarization of crustacean muscle fibers can be greatly delayed by tetraethylammonium and that the potentials can be made nearly square by tetrabutylammonium (Fatt and Katz, 1953a). Various smooth muscles, especially intestinal longitudinal and uterine muscle, show slow nearly sinusoidal waves. These may be independent of spikes or the spikes may arise from them (Holaday *et al.*, 1958).

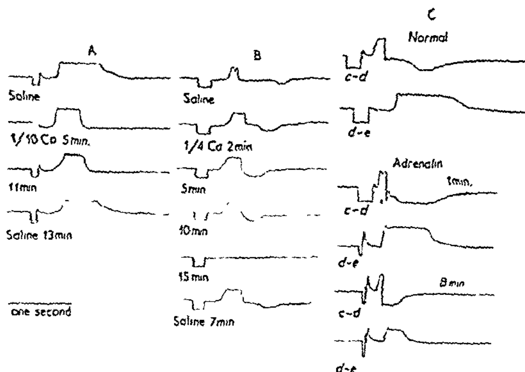


FIG. 18. Action potentials from rat ureter. A, monophasic; B, diphasic records, showing shortening of duration of depolarization by reduced calcium saline; C, series of diphasic (cd) and monophasic (de) records showing shortening of depolarization by adrenaline. From Prosser *et al.* (1955).

Whether th[illegible], plateaus, or some othe[illegible] established.

c. Condu[illegible] A variety of evidence indicates that conduction in smooth muscle can be independent of nerves. The velocity of conduction in the direction of the long axis of the fibers is 3 to 5 cm. per second or some 2 to 10 msec. per cell length. This speed, which is slower than in any known nerve fibers, has been found in smooth muscles of ureter, uterus, intestine, and chick amnion. Conduction can

norepinephrine inhibit activity of the external intestinal smooth muscles and are very likely involved in inhibition by sympathetic nerves. Conduction in the muscle is, however, not influenced by epinephrine. Serotonin (5-hydroxytryptamine) stimulates many smooth muscle preparations; it is without effect on interfiber conduction and its stimulating action in the intestine has been assigned to cholinergic postganglionic axons. In the taenia coli 5-HT produces an oscillation during which phases of excitation alternate with phases of blocked conduction (Bülbring and Burnstock, in the press). Serotonin also acts as a sensory stimulant to peristalsis when applied to the mucosa (Bulbring and Lin, 1958). Substance P is a polypeptide which stimulates intestinal activity; it is abundant in the mucosa, particularly the musularis mucosae, and also in the brain and in the prostate, but there is no indication that it might act as an interfiber transmitter (von Euler and Gaddum, 1931; Douglas *et al.*, 1951; Pernov, 1951; Eliasson *et al.*, 1956). Darmstoff appears to be a mixture of acidic phospholipids, found in intestinal extracts; it contracts rabbit duodenum, and its action is not antagonized by hexamethonium or cocaine. It does not stimulate a quiet cooled intestine which is stimulated by acetylcholine, hence it probably does not act on muscle or ganglia, but, like serotonin, on postganglionic nerve fibers (Vogt, 1953, 1956, 1957). Histamine is present in gastric and intestinal mucosa, much less in muscle. While chemical mediators between muscle fibers cannot be excluded, none of the known intestinal constituents seems to function as a transmitter.

It has been postulated that smooth muscle cells are connected by protoplasmic bridges and that a strip of smooth muscle is a syncytium. Possible bridges without crossing of myofilaments have been suggested for the uterus (Mark, 1956). However, many electron microscope observations of intestinal and uterine muscle fixed under different conditions have failed to reveal any true bridges; uninterrupted double membranes enclose all muscle fibers, although the outer membranes may approach within a few hundred Angstrom units of one another (Fig. 20) (Vatter and Prosser, unpublished; Cesar *et al.*, 1957). Bridges spanned by double membranes have been found in ureter (Bergman, 1958). If conduction between cells were by narrow bridges the current densities in the necks would be excessively large.

There might be electrical continuity or low resistance paths without histological bridges. This idea is supported by the facts that a demar-

nomic fibers at nearly 1 m. per second, while the muscle response travels only 3 cm. per second (Fig. 19) (Prosser and Melton, 1954).

It has been suggested without experimental evidence that the interstitial cells of Cajal might have some rôle in conduction (Jabonero, 1952; Koelle, 1954). The interstitial cells are abundant near non-myelinated nerve fibers in visceral muscle, they persist after deganglionation, they have long processes, and they are histochemically different from glia (Taxi, 1952). They contain nonspecific cholinesterase as do both glial and ganglion cells (Koelle, 1951). However, such cells are not found in conducting chick amnion and they appear to invest non-myelinated axons (Evans and Schild, 1953a; Cesar *et al.*, 1957). Although the function of interstitial cells is unknown, there is no good reason for implicating them in conduction in sheets of smooth muscle.

Several hypotheses of intercellular transmission may be considered. Smooth muscle is sensitive to mechanical stimulation, and pull of one cell might stimulate the next one. Excitability is enhanced by stretch. Microscopic observation of intestinal muscle shows some fiber movement even in stretched preparations. However, strips of circular muscle from cat intestine can be clamped locally so that no movement can be detected by a very sensitive lever beyond the clamp, yet full-sized action potentials pass through the clamped region; also, under such tension that no movement can be detected, propagated potentials are normal (Sperelakis and Prosser, 1959). In taenia coli, the frequency of spontaneous spikes decreases as tension is relaxed (Fig. 15) (Bulbring, 1955). It seems likely, therefore, that factors other than mechanical pull are necessary for interfiber transmission.

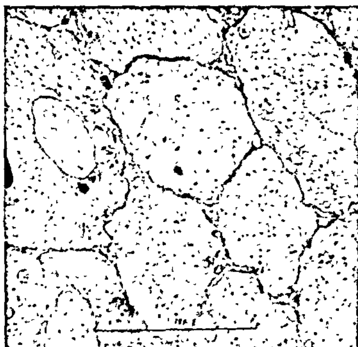
Transmission from one fiber to the next might be by a chemical mediator. Numerous agents are present in smooth muscle systems, particularly the intestine, which affect muscular activity. Acetylcholine stimulates the muscle cells directly; it is synthesized by the intestine, particularly in the mucosa (Feldberg and Lin, 1950). There is good evidence for cholinergic parasympathetic innervation of the intestinal muscle. Yet conduction per se in ganglion-free circular muscle is insensitive to acetylcholine, and also to eserine and to atropine. Velocity in the taenia coli is decreased by depolarizing agents such as acetylcholine and potassium. Acetylcholine may cause sustained depolarization by increasing permeability to Na, K and probably other ions; adrenaline may increase the membrane potential by stimulating the electrogenic Na pump. (Burnstock 1958 a,b). Epinephrine and

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A



B



FIG. 20. Electron micrographs of circular intestinal muscle of cat. A, cross section at low power; B, longitudinal section at higher magnification, note double membrane. Unpublished pictures by Dr. A Vatter.

cation potential can be measured from an injured end to the intact portion of a strip of smooth muscle, that a monophasic action potential can be recorded, and that spikes tend to be constant in height (Bozler, 1948). However, a variation in the response commonly occurs with repetition and with alteration in strength of the stimulus (ureter, intestine); frequently two patterns alternate in a series of successive responses (Sperelakis and Prosser, 1959). Measurements of impedance on strips of smooth muscles show not only reasonable membrane resistance but also capacitance. The specific membrane resistance of intestinal smooth

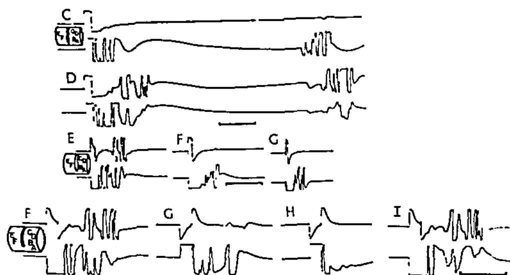


FIG. 21. Action potentials from ganglion-free circular intestinal muscle (cat). Ring of muscle slit over 85% of circumference, upper line in each record from post ring, lower from ring stimulated. C, no crossing of bridge; D, crossing after sodium citrate was applied to bridge; E-G, another ring; E, crossing of bridge; F-G, block after drop of isotonic KCl was applied to bridge (Time 1 sec.); F-I, another ring; H-I and I, polarity of stimulus reversed. From Prosser and Sperelakis (1956).

muscle fibers is similar to that of other muscles, about 1100 ohm cm² (Barr, in the press). Space constants appear to be very small, perhaps corresponding to individual cell lengths.

Transmission may be ephaptic from fiber to fiber (Prosser and Sperelakis, 1956). When a ring of circular intestinal muscle is mounted on a glass rod and slit on one side so that a narrow bridge of tissue connects the two halves, a spiralling wave of electrical activity from one ring can pass across the bridge to the other ring. Transmission across the bridge may be one-way (Fig. 21 F–I), but may be made to go in

both directions or may be enhanced in an inactive preparation by local treatment with calcium-free saline or with barium, that is, by agents which alter electrical excitability. Transmission is blocked by high potassium (Fig. 21 C–E). If the cut between the rings is made complete, so that the two muscle rings are separate, activity in the rings is independent when there is separation of 1 mm., but when they are pushed into close contact over a small portion of their circumference, signals cross from one ring to the other (Fig. 22). Transmission occurs across the

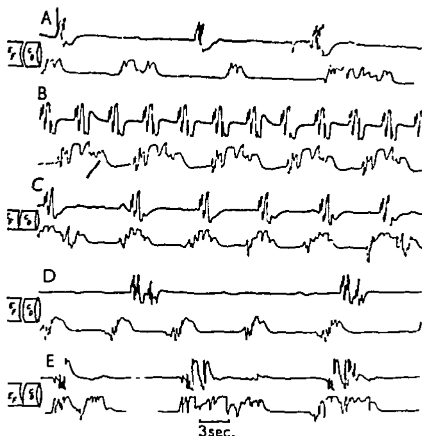


FIG. 22. Record from completely split rings of circular intestinal muscle, barium-induced rhythmicity. A, B, two rings separated by 1 mm., no synchrony; C, rings pushed in contact at bottom; D, ring separated; E, 50% zone of contact between rings. From Prosser and Sperelakis.

zone of contact in essentially the same way as across a bridge of tissue or in an intact ring.

The geometry of overlap of individual fibers is ideal for ephaptic transmission. There is some decrement in conduction over a long strip

of intestinal muscle mounted so that reinforcement by mechanical pull or myenteric reflexes cannot occur, but a wave can spiral around a plexus-free ring for several centimeters (Fig. 23). In addition to the conduction in the long axis of the fibers, there is some lateral influence from fiber to fiber. Electrical responses may occur out to a centimeter laterally when spiral conduction is prevented by slits or by local application of isotonic KCl. A wave of activity spirals around a ring of circular muscle in a band about 1–2 mm. or several hundred cells wide.

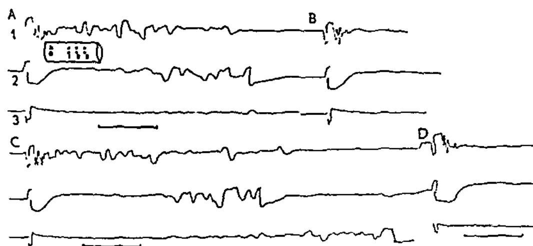


FIG. 23. Three-channel record of electrical responses to shocks in circular intestinal muscle. Stimuli at AB, channels correspond to electrode pairs 1, 2, and 3. Stimuli approximately 15 sec. apart. A, first stimulus elicits fast response at 1, spiraling response at 2, slight response at 3; B, fast (laterally conducted) response at 1 only; C, third stimulus elicits response at all electrodes; C fast response at 1 only. Time 1 sec.

Each fiber sets up an electrical field which influences and may activate the fibers lateral to it as well as those in its long axis. Responses fall off faster laterally than orthodromically. Knowledge of space constants and of impedance of individual cells is necessary before the effects of the local electric fields can be understood. For explaining transmission from fiber to fiber, the ephaptic hypothesis seems the most promising at the moment.

d. Regulation. Modifiability of excitability and regulation of activity by hormones and autonomic nerves is much greater in smooth than in striated muscle. Most mammalian visceral smooth muscles receive both parasympathetic and sympathetic innervation. A detailed analysis of

nervous regulation and the effects of drugs on smooth muscles is beyond the scope of this chapter. Drugs have been used extensively in efforts to reveal patterns of innervation and to identify excitatory and inhibitory mediators, yet the reported results are so confusing that generalizations are impossible. The confusion arises from the findings that (1) many drugs have more than one effect according to concentration, (2) a single drug may act at several sites within a muscle, (3) a given muscle may respond differently in different species, (4) different smooth muscles in the same animal differ greatly in their responses, and (5) a muscle may differ in its responses to drugs according to its physiological state. Some of the recent evidence for differences in drug and nerve effects may be cited, particularly to emphasize that one cannot generalize about mammalian smooth muscle. Examples are chosen mainly from intestinal and uterine preparations, a few from blood vessels.

The ganglion cells of the myenteric plexus mediate local reflexes, as indicated above. In addition, they serve as secondary neurons for parasympathetic nerves; these are excitatory and are cholinergic. Sympathetic axons to the intestine are inhibitory and adrenergic (von Euler, 1951, 1950); these axons come from secondary neurons of sympathetic ganglia, although recently it has been suggested that there may be either secondary sympathetic ganglion cells or inhibitory adrenergic neurons of the parasympathetic system in the plexuses. Acetylcholine stimulates intestinal muscle, even after ganglionic blockade, as do histamine and barium. However, higher alcohols block acetylcholine but not histamine stimulation, hence their action on the muscle is at different chemical sites (Geiger and Alpers, 1957). Both epinephrine and norepinephrine inhibit, and reports differ as to their relative effectiveness. Acetylcholine and epinephrine are normally antagonists. The lower ileum of the guinea pig, unlike the upper intestine, is contracted by epinephrine, an effect antagonized by nicotine (Munro, 1951; Vaughan Williams, 1954). In fetal guinea pigs, the ileum and sometimes the duodenum are contracted by epinephrine, an effect antagonized by nicotine (Munro, 1951; Vaughan Williams, 1954). In fetal guinea pigs, the ileum and sometimes the duodenum are contracted by adrenaline, a response which reverses with development (Munro, 1953). The muscularis mucosae of a dog, unlike external intestinal muscle, is contracted by both adrenaline and acetylcholine. The adrenaline effect is antagonized by ergot amines and the acetylcholine effect is antagonized by

atropine; sympathetic nerves excite the muscularis mucosae and inhibit the external musculature (King and Robinson, 1945). Also, aortal strips from rabbit are contracted by both acetylcholine and adrenaline. However, after dibenamine, adrenaline relaxes them (Furchgott, 1954, 1955).

Nicotine in low concentrations stimulates intestinal activity, and in high concentrations it paralyzes. The stimulating effect of nicotine is prevented by the cholinergic blocking agents hexamethonium and botulinum toxin (Ambache, 1951; Ambache and Lessin, 1955); nicotine stimulation is also blocked by D-tubocurarine, after which acetylcholine is still active (Ellis and Rasmussen, 1951). On the assumption that these blocking agents act on cholinergic ganglion cells, the site of the stimulating action of nicotine is assigned to parasympathetic secondary neurons (Youmans, 1952). After block of the stimulating action of nicotine, an inhibitory effect remains which is attributed (Ambache, 1951; Ambache and Lessin, 1955) to stimulation of adrenergic inhibitory ganglion cells. In cats and guinea pigs, but not in rabbits, atropine blocks the stimulation by nicotine (Ambache and Edwards, 1951). Deganglionated circular muscle from cat intestine is stimulated by nicotine at 10^{-5}, innervated intestine at 4×10^{-7} (Evans and Schild, 1953a). Also, the sensitivity of rabbit duodenum to nicotine is reduced by large amounts of acetylcholine and histamine, hence there must be some nicotine effect on muscle.

Barium is a powerful excitant for intestinal muscle and also for other smooth muscles such as the amnion. Yet in intact guinea pig ileum, stimulation by barium is reduced by hexamethonium, and hence barium must have some ganglionic action (Feldberg, 1951).

Atropine usually antagonizes the effect of acetylcholine and also of cholinergic nerves, but sometimes it antagonizes the drug but not the nerve. In the stomach, the stimulating action of the vagus is resistant to atropine in the dog but is blocked in cat and guinea pig (Ambache, 1955). Atropine antagonizes acetylcholine, but only partially blocks the effect of parasympathetic nerves on the bladder of the rabbit, even though the effect of stimulating the pelvic nerve is potentiated by eserine (Ursillo and Clark, 1956).

Serotonin (5-hydroxytryptamine) (5HT) has so many actions on smooth muscle structures that varied effects are not surprising. In the guinea pig ileum, serotonin stimulation is antagonized by atropine but not by hexamethonium or decamethonium, and also not by high con-

centrations of nicotine or by D-tubocurarine or by cocaine (Silva *et al.*, 1953; Gaddum and Hameed, 1954). Hence it is unlikely that 5HT stimulates ganglion cells or muscle, but it may act on cholinergic postganglionic nerve fibers. The stimulating action of 5HT on the rabbit gut and guinea pig ileum is not antagonized by lysergic acid diethylamide (LSD) whereas its constricting action on vessels of the rabbit ear and its stimulation of the uterus of the estrus rat are antagonized by LSD (Gaddum and Hameed, 1954). 2-Bromo-lysergic acid diethylamide (BOL) does, however, antagonize 5HT in the rabbit intestine and in the guinea pig ileum (Sollero *et al.*, 1956). In dogs serotonin causes a brief fall in blood pressure, followed by a prolonged rise and also a marked increase in cardiac output (Page, 1952); in cats, the predominant effect on blood pressure is that of a depressor. Also, in rats, after initial fall and then rise, low pressure is maintained. The depressor action of 5HT in cats is not antagonized by LSD or BOL, but in rats BOL reduces the effect of 5HT (Salmoiraghi *et al.*, 1957). In an isolated dog leg, 5HT constricts the blood vessels; when nerves are intact, the vessels are dilated (Page, 1952).

The responses of uterine muscle to drugs and to nerve stimulation varies with hormonal state. Estrogen can convert a uterus from a nonpropagating to a propagating muscle; progesterone decreases its excitability (Bozler, 1942b; Csapo, 1955; Jung, 1956). An estrogen-dominated uterus shows a positive staircase, that is, increasing tension on repeated stimulation by brief bursts of alternating current; under progesterone, the responses go in the reverse direction (Csapo and Corner, 1952). Oxytocin is a specific stimulant for a pregnant uterus; other smooth muscles are not affected by the reproductive hormones. Epinephrine relaxes a rat uterus, whether it be nonpregnant (and estrus) or pregnant, whereas in rabbits epinephrine contracts the uterus in either state (Balussa, 1940); in humans also, epinephrine contracts at all stages (Garrett, 1955). In the cat, however, epinephrine relaxes the nonpregnant uterus (estrogen dominated) and contracts the pregnant uterus (Morrison, 1940).

The cellular action of autonomic mediators on smooth muscle is virtually unknown. Adrenergic nerves liberate "sympathin" which is predominantly l-norepinephrine with varying amounts of epinephrine. Cannon's original idea, that excitatory or inhibitory effects result from E and I substances formed in the receptor cells in combination with a single mediator, is inadequate. The observed effects of adrenergic nerve

stimulation can be obtained in various tissues with l-norepinephrine and epinephrine; whether l-norepinephrine is excitatory or inhibitory depends on the receptor tissue (von Euler, 1950, 1951, 1956). However, the original evidence for diffusion through a muscle of an active agent formed on nerve stimulation remains unexplained (see p. 386). Most adrenergic nerves liberate much more norepinephrine than epinephrine, and usually, sympathin is considered equivalent to epinephrine. However, there are exceptions, such as the vasoconstrictors to the rabbit's ear, which liberate 8–30% epinephrine (Outschoorn, 1952). Many differences in sensitivity of effectors to norepinephrine and epinephrine, also changes in sensitivity with physiological state, have been described. For example, norepinephrine and epinephrine are primarily vasoconstrictor substances and raise blood pressure, but they dilate coronary vessels; pregnancy reverses epinephrine action in the cat uterus. The uterine region of the oviduct of a hen is relaxed by epinephrine and contracted by pituitrin, while the albumin-secreting and infundibulum regions are contracted by epinephrine, and not by pituitrin (McKenney *et al.*, 1931). In spinal cats or after acute denervation, the heart is accelerated by epinephrine more than by norepinephrine, whereas after chronic denervation the order of action is reversed (Innes and Kosterlitz, 1951).

The preceding examples of variation among smooth muscle systems could easily be extended; it is apparent that generalizations for various visceral smooth muscles and for different species are much more difficult than for striated muscles. The endpoints of measurement are usually very gross—change in amplitude, tone, or frequency of contraction—and the mere characterization of a drug or nerve as inhibitory or excitatory gives very little information. What is needed is to pinpoint the cellular sites of action and ultimately to identify the chemical receptors. Certainly the cellular sites and receptors are different in different smooth muscles. Since most visceral smooth muscles receive both excitatory and inhibitory innervation, it is possible that double innervation of some muscle fibers occurs; an alternative mechanism would be interfiber diffusion of mediators. Nerve endings on smooth muscle are poorly known; only recently (Cesar *et al.*, 1957) have electron micrographs shown endings terminating outside the muscle membranes, as in motor end-plates, rather than penetrating inside the muscle fibers as had been suggested by light microscopy. The effects of autonomic nerve impulses on cell potentials of smooth muscles have not been observed.

VI. CONCLUSIONS

The similarities among muscles in modes of activation are less striking than the differences. *Muscles cover a ten thousandfold range in speed* of movement. As Hoyle (1957a) has emphasized, a large part of this range is possible because of differences in activation mechanisms. Whether there are comparable differences in contractile proteins or in molecular histology of muscles is not known, but available evidence emphasizes similarities among actomyosins. Gradation of movement may be by number of motor units (e.g. vertebrate fast muscles), by frequency of nerve impulses (vertebrate slow), by selection of motor fibers evoking different speeds of contraction (crustacean and insect legs), by adding regional contractions due to small nerve fibers to ungraded contractions due to giant nerve fibers (squid mantle), by balancing peripheral excitation and inhibition (crustacean legs), or by tonic holding mechanisms (bivalve adductors). Muscle fibers may contract in response to their all-or-none impulses or to graded junction potentials; they may contract with little or no participation of the fiber membrane and they may even respond to repeated stretch of a resonating skeleton. Apparently, more muscles give graded rather than all-or-none responses, and all-or-none twitches may be a relatively recent evolutionary invention. Conduction in muscles may be along a muscle membrane in long all-or-none fibers, it may be by parallel nerve fibers, or it may be from short muscle fiber to fiber. The variety of nonstriated muscles in animals is great, and mammalian smooth muscles differ from one another so much that generalizations about "smooth" muscles are impossible.

Smooth muscles are sometimes considered primitive in that they show much independence of nervous activation and are slow. However, invertebrate nonstriated muscles, in which the fibers resemble smooth muscle histologically, show nervous conduction and are fast; visceral muscles of invertebrate animals related to probable vertebrate ancestors have not been studied from this viewpoint. It may be that the complex properties of smooth muscle represent relatively recent specializations. In few, if any, other types of tissue than muscle is there such variety of means to attain a function, each pattern adapted to its particular movement and each presenting a variation on a common theme of activation and contraction.

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CHAPTER IX

The Physiology of Muscular Exercise

Roger G. Bannister

I. INTRODUCTION

The physical capacity of an individual is limited by the speed of contraction of muscle on the one hand and by the force of contraction on the other. As a muscle approaches its maximum force, the velocity of contraction falls; the maximum force, for example, in weight lifting, is determined by the number and state of development of the active muscle fibers, which, in turn, are affected by the training to which the muscle has been subjected. At the opposite end of the scale, in sprinting and in throwing missiles, lies the effort in which speed, rather than force, is the decisive factor. Thus the balance struck between these two conflicting requirements, speed and force, depends on the nature of the exercise.

The aspect which will be considered more closely is the way in which muscular exercise is integrated, partly by means of chemical, physical, and nervous influences resulting from the muscular contraction itself. The particular facet of this integration strained by exercise, like the compromise between the speed and force of muscular contraction, varies with the nature of the exercise.

Running is peculiarly suited to a study of the factors involved in muscular exercise. Unlike many sports involving muscular effort, an absolute breaking point is reached, unaffected by those qualities of "touch" and "eye" which depend on neuromuscular co-ordination and make objective measurement difficult. Record performances in athletics may not often be adequately controlled, but they represent innumerable tests of maximal muscular effort. Consideration of the

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Running is peculiarly suited to a study of the factors involved in muscular exercise. Unlike many sports involving muscular effort, an absolute breaking point is reached, unaffected by those qualities of "touch" and "eye" which depend on neuromuscular co-ordination and make objective measurement difficult. Record performances in athletics may not often be adequately controlled, but they represent innumerable tests of maximal muscular effort. Consideration of the

limiting factors in running at different speeds throws valuable light on the physiology of muscular effort.

Hill (1925) was the first to plot speed against distance for world running records. Figure 1. shows the decline in speed as the distance increased, plotted on the basis of world records in 1955. Though Hill made no attempt to derive any mathematical equations correlating the decline in speed with the limiting factors responsible, others, Lietzke (1954) and Henry (1955) for example, have attempted to deduce the relationship between the physiological mechanism and the rate of

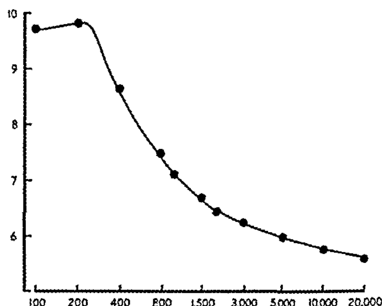


FIG. 1. World running records in 1955. Average speed plotted against log. distance.

energy liberation. Henry used three exponential terms characterizing muscle energy supply systems and has derived equations of motion from acceleration and fatigue factors. These equations describe the speed and position of a runner at any time, in steady pace or in "all out" running. But these studies are still empirical because there are insufficient data on rates of lactate accumulation and glycogen and fat depletion during "all out" running.

The quantitative assessment of the factors which are limiting might be made possible by a series of experiments in which the subject runs to exhaustion at different treadmill speeds. This would be a formidable task—for the subject as well as the experimenter—and has not so far been undertaken. Hence it is not possible to define precisely the speed

of running at which each limiting factor is maximally operative, but the nature of these factors during running at three different speeds will be discussed.

II. SPRINT RUNNING

In running short distances, oxygen uptake is unimportant because there is insufficient time for transport of oxygen from the lungs to the muscles. A sprinter may even with advantage hold his breath in a 100-yard race since a rigid thoracic wall provides the basis for the contraction of his limbs. Nor is the accumulation of chemical breakdown products limiting over very short distances, because speed does not fall as the distance run increases. Respiratory and circulatory adjustments are necessary only for the recovery, when products of the exercise are oxidized.

In sprinting, the body resembles a machine whose speed depends on the ratio of propelling force to resistance. Each foot draws and then drives the body forwards, the horizontal component of the thrust producing the runner's acceleration. The body is driven, as it were, by pendulums worked by muscular power instead of by gravity. By flexing the rear leg as it is drawn forward, the length of the pendulum is reduced so that the movement is completed more quickly and with less effort. Powerful arm movements are necessary to counterbalance leg movements. Ground friction and wind resistance may be estimated and inertia measured by the time taken to reach maximum speed. The total energy is mainly utilized in acceleration and deceleration of the limbs, overcoming their viscosity. The rate of energy expenditure while running at maximum speed is about 13 hp. for an average man. Fenn (1930) has calculated that the rate of effective mechanical work is about 3 hp., giving an efficiency of more than 20%.

III. MIDDLE DISTANCE RUNNING

Over the range of longer sprints and shorter distance races, there is a steady fall of speed with increase in distance. The supply of energy is partly aerobic and partly anaerobic; the accumulation of the products of muscular contraction is a major factor limiting the performance. Hill *et al.* (1924) suggested that the oxygen absorbed after the end of such exercise in excess of the resting oxygen uptake could be regarded as an "oxygen debt." By comparison with isolated muscle, lactic acid was thought of as the "security" given for payment of the oxygen debt.

There are several obstacles to accepting this theory in its simplest form. Margaria and associates (1933) have shown that no extra lactic acid appears in the blood during and after exercise involving oxygen debts less than about 2.5 liters. Also, there is no simple correlation between the oxygen usage after exercise, which falls rapidly, and the slow reduction in blood lactate, which is probably mostly taken up by heart and liver and to some extent by the muscles. The increased oxygen uptake after short maximal exercise is probably required to produce energy by oxidation of some compound or compounds (which may not be lactic acid) and resynthesis of the energy-rich bonds resolved by contraction and which may have accumulated in exercise.

In this range of muscular effort steady running may not be, as postulated by Hill, the most efficient. Runners seem to achieve their best times in the 440-yard race, for example, by running the first part of the race considerably faster than the second. Hill's constant speed hypothesis involves the assumption that the anaerobic and aerobic mechanisms of energy production are independent of one another. However, as lactate accumulates, the muscle pH falls, and it is possible that this in itself affects the efficiency of the aerobic mechanism.

IV. LONG DISTANCE RUNNING

As the distance increases, anaerobic mechanisms, although contributing a proportion of the total energy required, especially at the onset of exercise, become progressively less important. For example, anaerobic mechanisms probably contribute nearly half the total energy required in running a mile but less than a quarter of the energy required in running 3 miles. For longer distances, the work demanded of the muscle is within the range of speed and force which it can easily sustain. Such effort is limited mainly by the rate of supply of metabolic necessities to the muscle and the removal of the products of muscular contraction. Long distance running is of great physiological interest because it provokes the greatest respiratory and circulatory adaption of which the body is capable.

Analysis of the limits of performance in distance running requires an extension of our knowledge of steady state exercise. For equally efficient runners, the speed of movement depends on the maximal oxygen uptake which can be maintained. The blood lactate level falls during steady exercise (Bang, 1936), though this was disputed by Eskildsen (1947), and low steady muscle pyruvate and blood lactate

levels in excess of the resting values probably represent merely a new balance between rates of formation and removal.

During such exercise, the oxygen consumption of muscles (calculated from oxygen uptake in relation to body muscle weight) may increase some 50 times. Our knowledge of the effective limits of oxygen transfer from air to muscle is incomplete, but new light has been shed on some aspects of this problem. Recent experiments investigating the rise in ventilation, the oxygen transfer across the pulmonary epithelium, and the increase in cardiac output will serve to illustrate these trends.

A. The Rise in Ventilation

1. *Arterial pH and pCO_2*

The rise in ventilation during exercise was explained by Haldane and Priestley (1905) in terms of an increase in blood H^+ concentration and pCO_2. This view was challenged by Krogh and Lindhard (1917) on the grounds that the Haldane-Priestley method of alveolar sampling did not reflect the arterial CO_2 during exercise. Instead of sampling arterial blood during exercise, it is more practicable to infer the arterial pCO_2 from the alveolar air, because of the rapidity of sampling and the accuracy of analysis and also because the values so obtained may be calibrated by the use of the dead space dilution relationship. Using an end-expiratory sampling method, it was shown (Bannister *et al.*, 1954) that a rise of alveolar pCO_2 often takes place during exercise. The rise of alveolar pCO_2 in two athletic subjects was much greater than that in two unathletic subjects in whom emotional factors aroused by unaccustomed exercise may have contributed relatively more to the rise in ventilation. Asmussen and Nielsen (1956) have completed the calibration of the method and any error in the measurements was approximately cancelled out by the error of taking sitting values as a baseline for exercise. The view that the alveolar and arterial pCO_2 rises during exercise is at variance with that of Grodins (1950).

Any rise in alveolar pCO_2 which occurs in exercise is nevertheless quite insufficient to explain of itself the increase in pulmonary ventilation. There are three possible explanations for this. Firstly, carbon dioxide may not be a major factor in exercise dyspnea, as many have suggested (Comroe, 1944). Secondly, the sensitivity of the respiratory center to changes of alveolar pCO_2 (i.e. the response per unit change of stimulus) may be increased severalfold by some other factor. Thirdly, the threshold of the respiratory center to CO_2 (i.e. the level of the

alveolar pCO_2 during natural quiet breathing) may be lowered. Fortunately, there is a simple method of testing the third possibility. Avoiding the interference of oxygen lack by adding oxygen to the inspired air, we concluded that when the "effective" CO_2 stimulus (which includes the shift in threshold caused by acidosis) is estimated, ventilation and alveolar pCO_2 in exercise are related in a manner not unlike that found during CO_2 inhalation at rest.

2. *Temperature*

The effect of temperature is less easy to assess. Cotes (1955) showed an extreme rise of pCO_2 in subjects exercising when their body temperature was prevented from rising. He considered that there was no change in CO_2 sensitivity. Cunningham and O'Riordan (1956) have studied the effect of increase of body temperature by controlling the level of the alveolar pCO_2 so that respiratory alkalemia, which previously accompanied the hyperpnea, could be avoided. Below the normal alveolar pCO_2, sensitivity seemed to be reduced and this observation is compatible with the Danish view of threshold alveolar pCO_2 below which changes of pCO_2 do not affect respiration (Nielsen, 1936). At and above the normal pCO_2, however, sensitivity was approximately double that at ordinary temperatures. This contrasts with the oxygen breathing experiments of Cotes; during these conditions temperature probably acts by lowering the threshold to CO_2.

3. *Nervous Factors*

Nervous stimuli from the working limbs (Harrison *et al.*, 1932; Grodins, 1950) are probably of importance only in producing rapid adjustments to the changing conditions which occur at the start of exercise. Recently Åstrand (1956) has shown that, during the first 15 sec. of work on a bicycle ergometer, only a moderate difference in ventilation occurred in different experiments, despite an eightfold increase in the intensity of exercise and hence a considerable increase in "nervous activity."

4. *The Hypoxic Stimulus*

As the speed of running increases, there is evidence of another stimulus to respiration. Suddenly enriching the inspired air with oxygen produces a fall in ventilation with a very short latency. Asmussen and Nielsen (1946) postulated that a substance produced

by ischaemic muscles stimulates the chemoreceptors and is rapidly destroyed in the presence of oxygen. An alternative hypothesis in terms of known factors is that when oxygen is added to the inspired air, a chemoreceptor drive caused by arterial anoxemia is removed (Bannister and Cunningham, 1954). There are discrepancies between direct measurements by different investigators of arterial oxygen tension during heavy work. Lilienthal *et al.* (1946) found that it fell from 94 mm. at rest to 73 mm. during three experiments on Riley who exercised at moderately severe intensity but Mitchell *et al.* (1958) were unable to show a significant fall in arterial oxygen tension during exercise. Until the reasons for these discrepancies are elucidated, and they may be attributable to differences of method or exercise severity, it seems justifiable to refer to the "hypoxic" stimulus in the sense of a stimulus to ventilation which is removed by increasing the concentration of oxygen in the inspired air.

The experiments of Cormack *et al.* (1957) provide some indirect evidence for the hypoxic stimulus. These workers conducted rebreathing experiments at rest. They showed that in the presence of a large excess of CO_2, very powerful stimulation from oxygen lack may occur when the alveolar pCO_2 is well above 100 mm. and that the influence of these stimuli is not simply additive as Gray (1950) has suggested.

Thus there is some evidence that restriction of the supply of oxygen in severe exercise causes a further rise in ventilation when other respiratory stimuli are maximally active. Summarizing the chemical factors concerned in the rise of ventilation during exercise, it is likely that a rise of arterial pCO_2 does occur and that acidosis and temperature shift the threshold of the respiratory center to CO_2. With air breathing, the CO_2 sensitivity may be increased by the hypoxic stimulus and possibly also by a rise of temperature. Hence though ventilations at rest similar to those in exercise may be produced by the interaction of several stimuli, all probably operative in exercise, the qualitative interaction is as yet incompletely understood. The maximum ventilation, which varies with the individual but sometimes exceeds 100 liters per minute at S.T.P., is probably not limited by mechanical factors because it is considerably less than the maximum ventilation during forced breathing at rest.

B. Oxygen Transfer in the Lungs

For maximal oxygen uptake in the lungs, adequate ventilation during exercise must be balanced by adequate circulation of blood through the lungs. The effect of uneven distribution of blood and air to different parts of the lungs, suggested by Haldane and Priestley (1935), has been defined theoretically and measured by Riley and Cournand (1951), who expressed it in terms of a complete "venous shunt" which at rest probably amounts to 3% of the total blood flow. These advances have been made possible by new methods of blood gas analysis and by the technique of cardiac catheterization allowing direct measurement of the gas content of blood entering the lungs. Local over-ventilation and under-perfusion (and vice versa) increase the difference between the oxygen tensions of the alveolar air and the blood leaving the lungs. The smallness of the alveolar-arterial gradient at rest indicates the delicacy of the adjustment of alveolar ventilation to blood flow. It seems reasonable to assume that during maximal exercise this adjustment is to some extent sacrificed in favor of greater blood flow.

The diffusion capacity of the lungs for oxygen during exercise (the ratio of the gas transferred to the alveolar-arterial pO_2 gradient) has been measured in two ways. Riley and Cournand have used a direct method by lowering the inspired oxygen tension to the region of the blood dissociation curve, where changes of tension can be more easily measured. Bates (1955), adapting the CO method of Krogh and Krogh (1910), has recorded a similar increase in exercise. Riley and Cournand and also Bates explain the increase in diffusion capacity as due to an increase in the pulmonary capillary bed in exercise. In athletes, with a high diffusion capacity, Bates has suggested that changes in permeability of the pulmonary epithelium and capillary bed may play a part. This concept may require modification in the light of Roughton's recent work (Gibson *et al.*, 1955) on the kinetics of the reaction of CO with hemoglobin. He suggests that the formation of CO Hb. may be slow enough to retard the uptake of CO from the alveolar air. If this is so, more work will be required to assess the contribution of membrane resistance and the slowness of the CO Hb. reaction to the over-all observed limitation of diffusion as measured *in vivo*.

Recently the work of Lee and Du Bois (1955) has suggested a possible cause of the hypoxic stimulus during exercise. By enclosing a subject in a total body plethysmograph and measuring the uptake

of nitrous oxide by the lungs, they have succeeded in measuring changes in pulmonary capillary flow. They have shown that pulmonary capillary flow is pulsatile—the peak flow being 5 times the minimum flow. Transfer of N_2O, like that of CO_2, probably takes place at the beginning of the capillary. Their studies of oxygen uptake in the lungs are incomplete, but it is possible that when blood flow is maximal, as in exercise, oxygen, which diffuses more slowly, is incompletely taken up during the peak flow and a jet of blood with lowered oxygen tension passes from the lung capillary at each systole.

C. The Increase in Cardiac Output

If maximal oxygen transfer in the lungs is maintained, the rate of supply of oxygen to the muscle depends on the rate of circulation of the blood. Knowledge of circulatory control at rest is based to a greater extent on animal experiments than is knowledge of respiratory control. Knowledge of human circulatory control in exercise is therefore less advanced. There is now general agreement that the cardiac output, measured by the indirect Fick, foreign gas, or dye methods may rise to more than 30 liters per minute during severe exercise (Asmussen and Nielsen, 1955). In trained subjects the A–V O_2 difference remains low and constant at increasing rates of work and a large blood flow is maintained at low pulse rates because of the larger stroke volume (Christensen, 1931).

In studying the circulatory changes during exercise, it has been difficult to compare the results of animal and human experiments. Starling's theory of the increase in cardiac output in exercise, based on the heart lung preparation (Patterson and Starling, 1914), requires an increase in the diastolic volume of the heart. McMichael and Sharpey-Shafer (1944) have shown that there is a linear relationship between stroke volume and atrial filling pressure when the latter is varied passively. It seems, however, that during exercise in human subjects, the stroke volume may rise without an increase taking place in the diastolic volume (McCrea *et al.*, 1927) or the central venous pressure (Landis *et al.*, 1946). Ventricular function studies may provide the explanation for this apparent negation of Starling's principle. McMichael (1952) first suggested a series of different curves relating cardiac work to diastolic filling in human subjects, needed for normal, failing, and hypertrophied hearts. Sarnoff (1955), for anesthetised dogs with complete circulations, has plotted ventricular work against mean atrial

pressure (the indices which Starling's principle states to be directly related) on each side of the heart. Changes in the state of the myocardium produce different members of a family of curves but he has shown that a consistent relationship exists between atrial pressures and ventricular stroke work on the same, but not on the opposite side of the heart. Richards (1955) has pointed out that even a family of curves may be inadequate to describe the function of heart muscle when it is subjected to momentary changes of tone as a result of nervous, chemical, and hormonal influences, such as occur in exercise. The most satisfactory hypothesis appears to be that of Asmussen and Nielsen (1955) based on the work of Liljestrand and associates (1938); it suggests that the increased stroke volume during muscular work is produced mainly by more complete emptying of the ventricles during systole, called forth by nervous and possibly hormonal factors.

The reflex control of circulation during exercise is inadequately understood. Though impulses have been recorded from receptors which probably correspond to those formerly thought to initiate the Bainbridge reflex, the central effect of such afferents remains unknown. It is possible that there are reflexes arising in the right heart and pulmonary vessels which are sensitive to anoxia (Mills, 1944). Future work may elucidate the effect on respiratory and circulatory control of receptors in the heart and lungs which have been shown to cause profound changes in blood pressure, heart rate, and breathing (Dawes and Comroe, 1954).

D. Autonomic Factors

It is possible that the part played by the autonomic system in the smooth integration of exercise has been underestimated. The changes of blood flow to skin, splanchnic areas, and active muscles during exercise are well known. During exercise, however, the autonomic system also aids the regulation of body temperature. Heat liberated in contracting muscles, which increases the efficiency of these contractions, is at first retained; yet, when overheating occurs, heat loss mechanisms are augmented. While running on a treadmill, it is noticeable that the exercise becomes easier to maintain when the initial cutaneous vasoconstriction passes off and sweating occurs. A rise of rectal temperature to 105° F may never the less occur in strenuous distance running and high environmental temperatures may adversely affect performance (Bannister and Cotes, 1959).

In the future, it may be discovered that the sympathetico-adrenal system plays a part in extending momentarily the known limits of physiological adaptation. The effect of adrenaline injections in prolonging muscular exercise in fatigued dogs has long been known (Campos *et al.*, 1928). More recently von Euler (1952) showed that in a well trained human subject, noradrenaline excretion was slightly increased in moderate exercise. However, when the oxygen consumption rose above 2.4 liters per minute, the noradrenaline excretion rose to approximately 25 times the resting level. The stimulus to noradrenaline excretion in exercise, whether nervous or chemical, is unknown.

V. CONCLUSIONS

In conclusion, it seems that the muscular effort of running is limited by two factors. The first is the maximal contractile activity of muscle, which limits speed in sprinting. The second is the rate of supply of oxygen and metabolites to muscles and the removal of the products of muscular contraction. In steady state running, efficient oxygen transport to muscles is achieved by a physiological integration causing increases of pulmonary oxygen intake and transfer, cardiac output, and muscle blood flow. Yet in "all-out" running in which adequate carbohydrate is available, oxygen supply probably fails to keep pace with requirement. Oxygen supply is the weak link. But it seems academic to argue whether this failure occurs because the alveolar-arterial gradient for oxygen is increased or because blood is not circulating fast enough. The muscular effort of distance running appears to be limited by cardio-respiratory failure as a whole and not by premature failure of any single part of the integration.

Yet the difference between athletes lies not entirely in differences of cardiac output or diffusion capacity; it lies rather, I suspect, in their capacity for mental excitement, which brings with it an ability to overcome or ignore the discomfort, even pain, in muscles and the brain which is probably caused by ischaemia and the consequent changes of blood lactate concentration and pH. Though physiology may indicate respiratory and circulatory limits to muscular exercise, psychological and other factors beyond the ken of physiology set the razor's edge of defeat or victory and determine how closely an athlete approaches the absolute limits of performance.

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CHAPTER X

Part I. Some Effects of Fatigue, Temperature, and Training on Muscular Contraction in Man

O. G. Edholm

The effects of fatigue of voluntary muscle in man have been investigated by Merton (1954a, b), and the results have been described by the author in a recent review (Merton, 1956). The problem Merton has examined is a familiar one. When a muscle or a group of muscles are voluntarily contracted and relaxed regularly, provided that the force of contraction is at least half the maximal force, there will occur within a few minutes the onset of fatigue and the subject is unable by voluntary effort to contract the muscle at all. On the basis of animal experiments, it has been concluded that the site of such fatigue is mainly central and not peripheral, as maximal stimulation of the motor nerve of the fatigued muscle will still evoke a contraction, and it has been claimed that a similar effect could be obtained in man. Merton has studied the action potentials in a single muscle, which he has fatigued by means of a sustained contraction. When the muscle is so fatigued, stimulation of the motor nerve evokes the action potential, but no contraction of the muscle ensues. There is no recovery from the fatigue during ischemia, and recovery is gradual when the blood supply is intact. If the voluntary muscle contraction is performed with the circulation occluded, there is a steady decline in the tension exerted by the muscle as it fatigues. With the circulation intact, the decline in tension follows the same time course as in the ischemic muscle until the tension is reduced to approximately one-seventh of the original tension, when the rate of decline flattens out. These results show that fatigue is a peripheral, not a central phenomenon, and that the failure is not at the neuromuscular junction, since the muscle action potentials, evoked by stimulation of the motor nerve, are unchanged during fatigue. Merton suggests that the change in fatigue may be due to changes in the coupling between action potential and contractile substance. It has been shown by experiments on man that similar action potentials can evoke twitches which differ considerably, by slight changes in the synchrony of the exciting volley. The fatigue of muscles involved in skilled movements such as writing or piano playing has also been investigated by Merton. Occlu-

sion of the circulation affects writing after $1\frac{1}{2}$ min. and failure occurs after 2 min. There is no recovery with rest unless the condition is released. If the subject wrote for 2 min. before the circulation was occluded, then fatigue developed after 30 sec. of writing with ischemia. Merton concludes that there is little muscular reserve, and similar results have been obtained with writers and piano players. Not only is

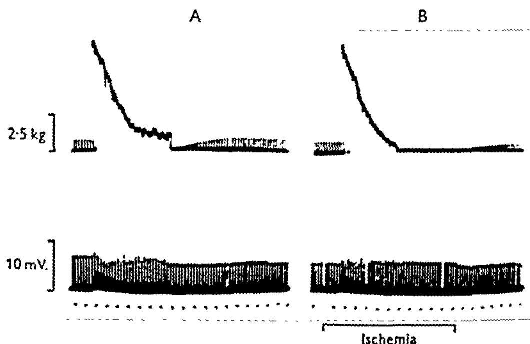


FIG. 1 The upper trace shows a series of twitches of the adductor pollicis muscle, evoked by maximal motor nerve shocks. These precede and follow a maximal voluntary contraction maintained until fatigued. A: Circulation intact. B: Circulation occluded during the period marked Ischemia. The lower trace shows the action potential, evoked by the motor nerve shocks. In the fatigued state the muscle does not respond to a motor nerve volley, and does not recover until the blood supply is restored. Fatigue is therefore peripheral in the muscle itself. From: P. A. Merton (1954) *J. Physiol.*, **123**, 553-564.

the muscular reserve in skilled movements surprisingly small, but the reason that this is not generally appreciated may be that muscular fatigue makes little difference to performance until the muscles are very severely fatigued. This suggested that the proprioceptive servo-control of muscular contraction (Hammond *et al.*, 1956) compensates automatically for fatigue.

A problem related to fatigue is the weakness of muscular contraction in a cooled limb. In part, the slowness of movement may be due to

increased viscosity especially of the synovial fluid (Hunter and Whillans, 1951; Hunter *et al.*, 1952). However, preliminary investigation has yielded some surprising results. Nukada (1955) examined the effect of immersion of the arm in hot and cold water on the time a sustained contraction could be maintained and found that the pre-cooled muscles took longer to fatigue than the pre-warmed muscles. Lind and Samueloff (1957) measured the fatigue time for sustained contraction of the forearm. The arm was kept in water for 30 min. at a temperature of either 34°C. or 18°C. Fatigue was more rapid at the higher water temperature. Successive determinations of the fatigue time were made at 7 min. and at 20 min. With the arm in water at 34°C., there was a decrease in the duration of sustained contraction with successive contractions, and the rate of decline was similar with intervals of 7 min. or 20 min. At 18°C., there was only a slight decline in the fatigue time when the interval between contractions was 20 min., but with 7 min. intervals there was a more rapid decline; after the third or fourth contraction, the fatigue time in the arm at 34°C. or 18°C. was similar.

The fatigue time was measured with the arm immersed in water at temperatures of 2°, 10°, 18°, 26°, 34°, and 42° C. by Clarke and associates (1957a). At 20° C., the fatigue time was very short, increasing with raised temperature until a peak was reached between 18–26° C. When the temperature was further increased, the fatigue time diminished again, and at 42° C. was as short as in the arm immersed in water at 10° C. It is remarkable that muscular contraction, measured in this way, appears to be more efficient at temperatures which are considerably lower than those normally existing in the body.

The hyperemia following these sustained contractions is directly related to the duration of contraction at water bath temperatures of 2° to 20° C. With the arm in water at 34° C. or 42° C., the hyperemia is considerably increased for contractions sustained for periods equal to those at lower water bath temperatures (Clarke *et al.*, 1957b).

These experiments on man can only be related with caution to observations on isolated muscles. In the rat ventricle (Feigen *et al.*, 1952) stimulation produced maximum contractions at 27.5° C., at 37° the contractions were only 60% of the maximum value. Szent-Györgyi (1953) has shown that the maximum tension in the rat diaphragm was developed at 27° C. Feigen (1956) comments that only a fraction of the total energy potential is being utilized at body temperature.

The effect of training on muscular performance is also related to the

problem of fatigue. A series of papers by Müller and Hettinger (Müller and Hettinger, 1954a, b, 1956; Hettinger, 1953, 1956; Hettinger and Müller, 1953, 1956) describes the effect of training in man on the maximum tension exerted by a muscle group. Training was accomplished by a series of contractions daily, either maximal or half-maximal or less. It was finally shown that improvement as judged by an increase in the maximum tension was achieved by training with contractions which exceeded 50% of the maximum. However, the amount of exercise required to produce a training effect was very small. One contraction a day, lasting for approximately 1 min., was sufficient to produce a training effect, which could not be exceeded by more frequent daily exercises. With daily exercise, increase in the maximum tension exerted continued for periods of at least a year. When exercises were done at weekly intervals only, there was also a training effect, but this developed more slowly. There was considerable variation between different muscle groups in training ability. At the end of a given period of time, the increase in maximum tension compared with initial values ranged from 37–181%. There appears to be a sex difference in that the ratio between male and female subjects before training was less than after training. Müller and Hettinger have also studied training in atrophied muscles.

More recently, Fletcher (1958) has given a preliminary account of experiments on training of the whole man. Subjects carried out the Harvard step test to exhaustion, every day. This test was designed so that most subjects would be nearly exhausted by or before completing 5 min. of stepping. Initial stepping times in Fletcher's experiments were 2–3 min. only. Little improvement was noticed in the first few days, but after 5–8 days the time to exhaustion began to increase, and stepping times of 15 min. were achieved after 20–30 days. The test is usually performed with one leg leading, i.e., the subject steps up with the right leg, and steps down with the right leg first. The left leg has a more passive role. When a stepping time of 15 min. could be accomplished with the right leg leading, the time to exhaustion using the left leg was measured. This was much shorter although greater than the stepping time with the right leg on the occasion of the first trial. If the stepping time with the right leg was initially 2½ min. and after 20 days, 15 min., then the time with the left leg might be 5 min. The resting heart rate before and after a training period showed a significant decline. In all these experiments, care was taken to ensure that the subjects did not

change their ordinary mode of life, in particular that they did not take more or less exercise. There are some interesting similarities between the results of Müller and Hettinger and those of Fletcher, and in particular in both cases a very brief period of muscular activity has an effect which persists for one or more days. The rate of decay has been examined in part by Fletcher, but in more detail by Müller and Hettinger. Initial rate of decay can be fairly rapid, but takes a long time before the initial base line is reached. Part of the difficulty of these experiments is to determine the time course, as it may take many months before training reaches a maximum, and possibly longer before it returns to control values.

In all the experiments on man briefly described in this section, simple or relatively simple techniques have been used. It is clear that here is a field of investigation in which the detailed physiological and biochemical mechanisms underlying these phenomena will require much intensive work to unravel.

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CHAPTER X

Part II. Fatigue of the Working Individual

E. Hohwü Christensen

I. WORK LOAD, CAPACITY FOR WORK, AND FATIGUE

Fatigue is a term used to cover all those determinal changes in expression of an activity which can be traced to the continuing exercise of that activity, and which can be shown to lead, either immediately or after delay, to deterioration in the expression of that activity (Bartlett, 1953).

Fitness consists in the ability of the organism to maintain the various internal equilibria as closely as possible to the resting state during strenous exertion and to restore promptly after exercise any equilibriums which have been disturbed (Darling, 1947).

The factor of safety for man may be defined as the ratio of the maximal increment in metabolic rate to the observed increment (Dill *et al.*, 1936).

Machines are not usually run at more than 50% of their capacity, and a similar safety margin should perhaps be allowed to man and animals so as to avoid untimely death (Brody, 1945).

The "50% load" must be related to the capacity of the involved muscles and not to that of the body as a whole. When increasing the metabolism over this 50%, the feeling of discomfort and strain will probably be marked (Åstrand, 1952).

If the above mentioned statements are correct—and there are good reasons to believe they are—fatigue and fitness are closely interrelated. Deterioration in a certain activity due to disturbances in the various internal equilibria depends on the ratio between the work load and the

fitness or capacity of the working individual as a whole or of that part of the individual which is most closely loaded towards its maximal capacity and which consequently has the narrowest margin of safety.

Skeletal muscle has an unique capacity for increasing its rate of metabolism. While the resting muscle has an O_2-consumption of less than 2 ml. per kilogram per minute the muscle at maximal work can consume some 200 ml. per kilogram per minute with a fiftyfold increase in blood circulation compared to the resting state (Asmussen *et al.*, 1939). Through anaerobic processes, a many times higher rate of energy delivery is possible for a short spell of time.

The internal equilibria of the working muscles depends primarily on the blood circulation. The blood carries oxygen and fuel to the muscles and eliminates CO_2 and other waste products as well as heat. An adequate blood supply is, therefore, the most important problem for a stable internal equilibrium and the great majority of fitness tests in reality test the capacity of the circulatory system (Åstrand, 1956). Many industrial jobs place a heavy load on relatively small units of skeletal muscles. This load may not influence the factor of safety of the general circulation but may well do so for the local blood supply. In this case, general fitness tests will give little or no information about the factor of safety.

II. ANAEROBIC WORK AND FATIGUE DUE TO "INTOXICATION" BY WORK PRODUCTS

In spite of changing opinions about the role of lactic acid in muscular contraction, it is an undisputable fact that blood lactic acid concentration, unchanged during light work, increases when work intensity rises above a certain level. This level is not an absolute one but depends on the fitness of the subjects (Dill *et al.*, 1930). A fit subject with a maximal oxygen intake of 4 or 5 liters per minute does not necessarily show any increase in blood lactic acid at work loads requiring less than 2.5 to 3 liters, whereas a less fit subject will show a marked increase. Parallel with an increase in blood lactic acid, a greater oxygen debt is found, indicating an insufficient oxygen uptake during actual work (Margaria *et al.*, 1933). After short bursts of maximal activity, as in a 400 meter race, blood lactic acid concentration will reach values of 150 to 200 mg.% (10 mg.% at rest) and practically the whole extra oxygen intake will appear as oxygen debt. Here are good reasons to believe that the lactic acid concentration in the blood is a good indicator of

insufficient oxygen supply to the working muscles. As long as the oxygen tension in the muscles is above a certain level, the blood lactic acid and possibly even the muscle lactic acid concentration is normal, with no disturbances in the internal equilibrium. If the demand for oxygen increases above a certain level, the oxygen tension drops due to a disproportion between oxygen usage and supply from the blood, and anaerobic processes with lactic acid as a final state will take over a larger fraction of the muscle metabolism. A sensation of strain or fatigue is closely related to the blood lactic acid concentration, and lactic acid can be looked upon as a "fatigue toxin" in many instances. Parallel with an increase in muscle and blood lactic acid concentration, pH changes will occur and the alkali reserve of the blood will diminish. Other anaerobic byproducts of muscle metabolism will also contribute to disturbances of the internal equilibria. If a heavy load is placed on small units of skeletal muscles, the total amount of lactic acid may not be sufficient to produce significant changes in the mixed venous blood, in spite of high local concentrations in the muscles and in the venous blood leaving them. In these instances, the lactic acid concentration of the mixed blood is a very poor indicator of fatigue or strain.

Trained athletes seem to tolerate higher lactic acid concentrations than untrained, and children and old people tolerate less than adults (Robinson, 1938). No definite sex difference in this respect seems to exist (Åstrand, 1952).

III. FATIGUE DUE TO DEPLETION OF FUEL

The fuels used in rest as well as in work are essentially those supplied by the diet. When working in a fasting state, the fuels used are dependent on their relative availability and on the intensity of work. A well trained, fasting subject, who for a week had lived on a fat-rich and carbohydrate-poor diet (R.Q. in rest 0.75), was able at work to maintain a tenfold increase in metabolic rate, with fat supplying more than nine-tenths of the energy. After 90 min. of work he was totaly exhausted. Only 35 g. of carbohydrate were metabolized during the work period, but low blood sugar values indicated that the carbohydrate stores nevertheless were depleted. Living for a week on a carbohydrate-rich diet (R.Q. in rest of 0.96), the same subject could in a fasting state work for 240 min. at the same work rate, oxidizing not less than 400 g. of carbohydrate, before exhaustion (Christensen and Hansen, 1939a).

In experiments with dogs running on a tread mill, it could be shown

that ingestion of carbohydrate during the work period would increase the running time two- to threefold. A totally exhausted dog with blood sugar values of 46 mg.% was able to go for 75 min. more after ingestion of 50 g. of sugar (Talbott *et al.*, 1932). Similar results were obtained in man (Boje, 1936). Recent results with trained, fasting subjects, exhausted during skiing, support very definitely the opinion that the available glycogen in a trained man living on a carbohydrate rich diet amounts to some 400 g. (Hedman, 1957). Of further interest in these experiments was that the skiers, who had to maintain a constant high running speed requiring an oxygen intake of 4 liters per minute, kept an almost constant R.Q. close to 0.9 from start to finish. Here the R.Q. was determined by the intensity of work. At low and at moderately high work intensity, R.Q., as mentioned above, will depend on the relative availability of carbohydrate stored as glycogen in liver and muscles. As work goes on and the stores are reduced, R.Q. drops (Courtice and Douglas, 1936).

Above a certain work intensity, where the margin of safety for the oxygen supply to the working muscles is severely reduced, a high carbohydrate metabolism is apparently necessary even if the glycogen stores are more or less depleted. The explanation might be that for a given quantity of energy transformed, carbohydrate requires less oxygen than does fat, and that the production of mechanical energy is more efficient from carbohydrate than it is from fat (Krogh and Lindhard, 1920). Even at a moderately high work intensity, a fit subject can for a long time rely on his body fat as an energy depot, if, through the ingestion of small amounts of carbohydrates, hypoglycemia and an accumulation of acetone bodies can be avoided. With an ingestion of a total of 250 g. of carbohydrate, corresponding to 1000 cal., two trained skiers were able to go on for 3 days with a total caloric output of 18.000 cal. (Åstrand and Hedman, 1954).

The present results indicate that the main cause of fatigue in work experiments with small carbohydrate depots is a too low glucose level in the central nervous system and not primarly a lack of fuel in the working muscles (Christensen and Hansen, 1939b). To what extent a diminished glycogen content of the working muscles may reduce the capacity for anaerobic work is still an unsolved problem, but there are experimental findings which indicate that this might be the case (Hedman, 1957).

IV. ENVIRONMENT AND FATIGUE

A. Low Oxygen Tension

An adequate oxygen supply to the working muscles is, as already mentioned, one of the most important prerequisites for an undisturbed equilibrium. Quite naturally, therefore, a reduction of the normal oxygen tension (160 mm. Hg) of the inspired air reduces the working capacity for more sustained work, while work of very short duration may be more or less unimpaired (Margaria, 1929; Christensen and Nielsen, 1936). The limits for maximal aerobic performances are reduced at altitude both in unacclimatized and in fully acclimatized subjects. It is still an unsolved question, however, which link in the oxygen supplying mechanisms has the narrowest margin of safety at altitude.

In an unacclimatized subject at work, both lung ventilation and minute volume of the heart will be higher than normal. During acute exposure to a simulated altitude of 12,000 feet a lung ventilation of nearly 200 liters per minute (B.T.P.S.) was found at maximal work (Åstrand, 1954). For a given submaximal work load, the heart rate may be 30–40 beats per minute higher than with a corresponding load at sea level, indicating a higher circulatory rate partly compensating for the diminished oxygen content of the arterial blood (Christensen and Forbes, 1937). Even the blood lactic acid values for a given work load are higher at altitude than at sea level (Dill *et al.*, 1931; Lundin and Ström, 1947), and maximal concentration for blood lactic acid may occur at a load that hardly gives any increase at sea level (Asmussen *et al.*, 1948).

In marked contrast to these values, obtained in unacclimatized subjects during acute exposure to altitude, are the results obtained on partly acclimatized man. The heart rate, which in maximal work at sea level was 190, dropped to 135 beats per minute at 15,000 feet after several months of acclimatization to altitude (Christensen, 1937). And in spite of the fact that the aerobic work capacity was reduced to half of the sea level value, a given grade of work did not produce a greater than normal increase in blood lactic acid. The maximal concentrations for blood lactic acid were consequently far below those obtained in maximal work at sea level (Edwards, 1936). Only the lung ventilation showed the same or practically the same maximal values at all altitudes and this may possibly be the most important limiting factor for work at altitude (Christensen, 1937).

that ingestion of carbohydrate during the work period would increase the running time two- to threefold. A totally exhausted dog with blood sugar values of 46 mg.% was able to go for 75 min. more after ingestion of 50 g. of sugar (Talbott *et al.*, 1932). Similar results were obtained in man (Böje, 1936). Recent results with trained, fasting subjects, exhausted during skiing, support very definitely the opinion that the available glycogen in a trained man living on a carbohydrate rich diet amounts to some 400 g. (Hedman, 1957). Of further interest in these experiments was that the skiers, who had to maintain a constant high running speed requiring an oxygen intake of 4 liters per minute, kept an almost constant R.Q. close to 0.9 from start to finish. Here the R.Q. was determined by the intensity of work. At low and at moderately high work intensity, R.Q., as mentioned above, will depend on the relative availability of carbohydrate stored as glycogen in liver and muscles. As work goes on and the stores are reduced, R.Q. drops (Courtice and Douglas, 1936).

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oxygen intake (Christensen, 1953). The extra 62 heart beats per minute were due to the heat load, and the subject's capacity for oxygen transport was consequently severely diminished.

Dehydration due to excessive sweating at work in a hot environment may contribute to an increase in heart rate and in rectal temperature. The effects of diverse stresses when working in a hot and humid environment are almost strictly additive and resulting strains are cumulative. An increase of 10° (F.) air temperature (above skin temperature) increases man's circulatory strain as much as energy expenditure of 29 cal. per square meter per hour. A dehydration of 1% of body weight increases circulatory strain as much as an energy expenditure of 24 cal. per square meter per hour (Brown and Towbin, 1947).

High temperature and severe dehydration may not only have a deleterious effect during work, but even the maintenance of standing posture can become a severe problem. Blood pooling in the limbs may reduce the effective circulation blood volume to such a degree that the blood pressure at the level of the brain become insufficient for the oxygen supply of the brain cells and syncope ensues (Rothstein and Towbin, 1947). In less severe cases, a sensation of restlessness and severe fatigue may be the result of these unfavorable conditions.

V. REST PAUSES AND FATIGUE

A well trained subject worked on the bicycle ergometer with a work load of 2160 kpm/min. to complete exhaustion after 9 min. The blood lactic acid rose to 150 mg.%, the pulmonary ventilation (V_{37}) reached 124 liters per minute, the final pulse rate was 204, and the oxygen intake was 4.61 liters per minute. If the same subject worked discontinuously with alternating periods of work (2160 kpm/min.) and rest, the experiment could be carried on for 1 hour or more, depending on the length of the intervals. With half-minute intervals of work and rest, the blood lactic acid increased insignificantly to a level of 20 mg.%, V_{37} was below 65 liters per minute, the pulse rate was below 160, and the oxygen intake never exceeded 2.9 liters per minute. These values were fairly close to values obtained when the subject worked continuously with half the load. After 1 hour, the subject did not feel at all tired. When the length of the intervals for work and rest were increased to 2 or 3 min., a corresponding increase of strain and stress was felt by the subject. With 3-min. intervals of work and rest, the experiment could be carried through for 1 hour only with the greatest diffi-

B. High Oxygen Tension

At sea level, an increased O_2-tension in the inspired air will have no or a very slight effect in rest or at low work intensities. If, however, the work load comes close to the upper limit for aerobic work, a marked decrease in lung ventilation can be seen and the oxygen intake per minute can surpass normal sea level values. A trained subject could increase his maximal oxygen intake from 4.20 liters up to 5.05 liters per minute when normal inspired air at sea level was substituted by an air mixture with 45% oxygen (Nielsen and Hansen, 1937). At altitude, the inspiration of 100% oxygen can completely eliminate the effect of altitude up to highest summits that can be climbed, if no acclimatization has taken place. If the mountaineer is acclimatized to altitude, oxygen breathing will improve the working capacity very definitely but will not fully restore it to normal sea level values (Åstrand, 1954).

C. High Temperature

The rectal temperature increases linearly with the rate of metabolism (work load). Rectal temperatures of 38° to 39°C. are to be expected whenever the O_2-uptake for some length of time is 3 liters per minute or more. This is by no means an indication of insufficiency of the heat regulation, as the rectal temperature of a naked subject at a certain work load was found to be independent of the environmental temperature between 5° and 35°C. and a humidity of 50% (Nielsen, 1938). This change in the internal equilibrium at a state of high metabolic rate has apparently a beneficial effect as far as the working capacity is concerned (Asmussen and Böje, 1945) and should not be looked upon as a symptom of fatigue.

At very high intensities of radiating heat or in a hot and humid climate, the heat dissipation at work will, however, be insufficient and fatigue may arise in a relatively short time. There will be a continuous rise in rectal temperature, and what may be even more disastrous, a marked rise in skin temperature. Due to reduced cooling of the blood circulating in the skin vessels, the circulatory rate will have to increase, and a large blood volume will be "stored" in the skin. The heart is forced to work with a small stroke volume and at a high pulse rate (Dill, 1936). A trained subject, when working in a normal environment, had a heart rate of 104 at an oxygen intake of 1.4 liters per minute; at high radiating heat, the heart rate was 166 at the same

oxygen intake (Christensen, 1953). The extra 62 heart beats per minute were due to the heat load, and the subject's capacity for oxygen transport was consequently severely diminished.

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culty, and at the end of the hour the subject was completely exhausted. The blood lactic acid after the first 15 min. was at a level between 115 and 125 mg.%, V_E was between 105 and 110 liters per minute, the heart rate was about 190, and the oxygen intake reached the maximal value of 4.6 liters per minute.

As all experiments lasted for 1 hour, and as always the actual working time was 30 min., the same total amount of work, 64,800 kpm, was always completed. However, the degree of fatigue, subjectively felt and objectively measured (through the blood lactic acid concentration), varied enormously (Christensen, 1956). In experiments with half-minute intervals, the rest pauses could obviously contribute to a more or less complete restoration of the disturbed equilibria. With intervals of 3 min. the disturbances were more severe and could never be restored during the 1 hour experiment.

Extensive research work on the influence of rest pauses on fatigue in industrial work has been done during recent years (Lehmann, 1953). Changes in pulse rate have been used as a convenient indicator of general as well as local fatigue (Müller, 1955).

VI. RECREATION, PHYSICAL TRAINING, AND FATIGUE

The purpose of recreation should always be to establish a state of personal well being. Recreation is not necessarily a state of inactivity; it involves an agreeable occupation, usually different from the ordinary work. The subjective motivation for *active* recreation is to enjoy the use of certain capabilities for fun and pleasure. From the point of view of fatigue, one would perhaps expect that the worker, who during his work day is occupied in heavy labor, would choose recreational activity which involves little or no physical stress. This is, however, not at all typical. The Scandinavian ski elite, for instance, is almost exclusively recruited from forest workers. They may during the winter use skis as a means of transportation in their daily heavy work, but at night or over the week end participation in ski competitions will be their favorite recreation, just as playing socker will fulfill the same function during the summer.

Semiprofessionalism is a great danger to several branches of recreational sport activity. In professional or semiprofessional sport, the recreational point of view may be lost entirely for the participants themselves; such events should be evaluated only as passive recreation of the spectators.

There are good reasons to believe that active recreation in many instances improves the worker for his job and makes him less fatigable. The intensity of "work" is ordinarily much higher during sport activity than in professional labor. In forest work, usually regarded as one of the heaviest professions, an oxygen intake of 2 liters per minute will be close to the upper limit (Lundgren, 1946). In skiing, for instance, 4 liters per minute is quite usual. During skiing, the forest worker will increase his work capacity for aerobic work, his margin of safety will be widened, and he will be less fatigable in his ordinary job. In a study of recreational participation of workers in industry, a high correlation between participation and industrial efficiency was found (Creed, 1946).

With the increasing number of motor cars, elevators, escalators, and so on, the physical activity of daily living may be reduced to such an extent, that without active physical recreation, the standard of physical fitness of the general population will be so low, the margin of safety so narrow, that any extra activity may induce fatigue and overstrain. The very instructive results obtained after 3 weeks of complete physical inactivity (bed rest) in a number of healthy subjects show more than anything else the deleterious effect of inactivity on work performance (Taylor *et al.*, 1949). Systematic physical training of patients with low physical work capacity resulted in a marked improvement after 6 weeks of training (Holmgren *et al.*, 1957).

The fact that active recreation or physical training improves the capacity for sustained muscular work and decreases fatigability may be due to improvements of the oxygen transport system, increased muscular strength and improved coordination and efficiency. It may also be due to an adaptation of the hormonal systems to exercise. The adrenal cortex especially may play an important role in this respect. Muscular exercise is usually rated as a factor of stress, and in an untrained individual a certain intensity of muscular activity may result in a depletion of the precursor substances of the glands, with all its consequences for the individual. Through systematic training, the glands may show the usual response to gradual persistent stress.

Physical exercise can result in fatigue but it should be rated as a most valuable antidote against fatigue in daily living. Sufficient rest periods and undisturbed sleep of sufficient length should, of course, always be included in any rational scheme against fatigue and for the preservation of physical and mental capabilities.

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CHAPTER XI

The Control of Muscular Activity by the Central Nervous System

MARION HINES

I. INTRODUCTION

Reluctance to credit the central nervous system with control of skeletal musculature marked the attitude of the public and of physicians for centuries. Severe head injuries were not correlated with ensuing change in the use of skeletal muscle. For example, during the siege of the Castle of Birr, the Earl of Kildare received a bullet wound which produced partial paralysis and difficulty with speech. He was called to England by the ministers of Henry VIII to defend himself against an accusation of working with the Catholics in Ireland. In contrast to his previous fluent defense before Wolsey, his speech was slow and hesitating. This hesitation was interpreted as guilt rather than attributed to aphasia. Later, even Charcot met with skepticism when he presented his correlations of changes in the use of skeletal muscle with lesions of the central nervous system. The Royal Society denied

these two divergent results continued to be interpreted as the sequela to injury of or loss of a single corticifugal system.

The single corticifugal system was described as stemming from large pyramidal cells (Holmes and May, 1909) in the Vth layer of Brodmann's area 4 (1903) and as penetrating the spinal cord, thus forming the tractus corticospinalis. The demonstration that this corticifugal system was interrupted in paralysis of cortical or of capsular origin required years of correlation of clinical and pathological findings (Charcot and Pitres, 1895). The modification of that correlation has been equally difficult. Other origins for this system, such as the postcentral gyrus (Vogts and Sachs, see Foerster, 1923) and the pyramidal cells in the IIIrd layer of area 4 (Spielmeyer, 1906) were long ignored, just as were other corticifugal systems such as the corticopontile (Dejerine, 1901), the corticothalamic (Dejerine, 1901; Vogt, see Foerster, 1923), the corticorubral, and the corticonigral (von Monakow, 1909, 1910). Indeed, the motor cortex became and remained limited for many years to the precentral gyrus in spite of Ferrier's (1876) record of motor activity obtained by electrical stimulation of the parietal, temporal, and occipital lobes.

If the newer implications of the control of tone had been accepted by the majority of clinical neurologists, Bucy's recent impassioned appeal (1957) for discard of the allocation of spastic paralysis to "an upper neuron lesion" would have been unnecessary. Neurologists are after all as conservative as the tissue they study.

In this study, therefore, the phasic and tonic activity of the control of skeletal muscle will be related to the fiber tracts and their cells of origin, not only within the central nervous system but in the peripheral as well.

II. NORMAL USE OF SKELETAL MUSCLE BY THE PRIMATE

The ability to control the contractions of skeletal muscle which characterizes any particular animal depends upon many diverse nerve fibers within the peripheral and central nervous systems. This control is of course initially dependent upon both the sensory and motor innervation of these muscles. The sensory innervation of skeletal muscles in the mammal is found upon specialized muscles, the intrafusal fibers of muscle spindles, upon the tendons, and about the tendinous attachments of the extrafusal fibers. The motor innervation of muscle fibers attached to bones in mammals is generally simple; a single terminal is

Marshall Hall the privilege of publication of his later studies upon the central nervous system of small reptiles and amphibians, because they were reluctant to accept the presence of the central nervous system as indispensable for a motor response to a sensory stimulus.

On the other hand, Magendie's experiment met with no such antagonism. That the dorsal roots "were destined for sensation" and that the ventral roots carried motor impulses easily became a part of the body of neurological fact. It was many years (1860) before Brongeest showed that the hind legs of the frog became limp and toneless after the dorsal roots of the lumbar plexus were severed homolaterally. It remained for Sherrington, however, *to demonstrate conclusively that* the nerve fibers in the dorsal roots which maintained tone in skeletal muscle were distributed with the motor nerves which innervated skeletal muscle.

The spastic state which accompanied hemiplegia in man was not recognized as such until the 1870's. The facets of this state include brisk and irradiating tendon reflexes, increase in resistance to passive movement of the clasp-knife type, and clonus. Spastic hemiplegia in man, most frequently the result of hemorrhage into the internal capsule, left at death only one lesion in the spinal cord. This lesion was confined to a loss of nerve fibers in the dorsal half of the lateral funiculus and in the ventral funiculus, the sites of the pyramidal tract. Thus it was that the spasticity and the paralysis were assigned to the loss of a single system.

Although Hughlings Jackson did not allocate this dual change in skeletal muscle following such severe injuries to particular fiber tracts within the central nervous system, he did (Vol. II, pp. 3-39 of Selected Writings) consider that "the process of dissolution is not only a 'taking off' of the higher but at the same time a 'letting go' of the lower." Thus, not only were certain normal functions lost, but also other functions were released, due not to irritation by pathological processes, but rather to loss of control over lower levels of the central nervous system by its higher ones.

Therefore, in spastic hemiplegia in man, the functions lost would be the skilled movements of the muscles of the extremities contralateral to the lesion and those which were "let go," (the phenomena of release, Walshe, 1929) the hypertonus, the briskness of the tendon reflexes, the exaggeration of reflexes of labyrinthian origin, associated movements, and the positive Babinski. In spite of Hughlings Jackson's suggestion,

Two modes of organization distinguish the use of skeletal muscle, namely, mass organization and discrete organization (Tower, 1940). In the former, the innervation begins in the proximal muscles and flows down the limb to involve successively more distally lying muscles, either as extensor or as flexor sheets, the extensor or flexor synergies of Foerster. This mode of innervation in the intact primate is preceded by some degree of fixation of the muscles of the girdles and of those which hold the extremity to the girdle. Discrete organization is distinguished by innervation of distally lying muscles, which, as the movement progresses, may either include more proximally lying muscles in active contraction or be accompanied by fixation of more proximal muscle groups, including those of the girdle to which the extremity is attached.

The discrete use of skeletal muscle, directed toward an object desired by the primate, is the complex resultant of interdependent activity of many muscle groups employed in definite and distinct ways, capable of partial analysis. Discrete control of skeletal muscle requires ease of initiation of the contraction, a grading of contraction either simultaneous or consecutive, the ability to stop this contraction at any phase of its execution, and to start again in the same phase or to redirect the aim of the whole movement. An enduring substratum of posture is a prerequisite of such activity; for posture must be maintained in an easy and natural way to free the hand and upper extremity for the examination of objects or the manipulation of tools, and to anticipate by adjustment of the muscles of the trunk and girdles the next stage of directed movements. The variants in cooperation which make skilled movements successful are fixation of proximal musculature and increment and decrement of tone in muscles of the trunk, in those which attach the extremities to the girdles as well as in those which lie proximal to the actively contracting muscle groups. In the attainment of skilled muscular activity, these facets of use of skeletal muscle of the trunk or girdles are as important as the phasic activity of the muscles of the digits of the hand.

Furthermore, skilled movements require participation of both upper extremities, the one which leads and the other which cooperates. The activity of the muscles of the leading extremity is initiated distally, that of the cooperating extremity, proximally. The manner of use of the muscles within each of the two extremities is dissimilar. In Beevor's (1903) analysis, movement by the leading extremity is initiated by prime movers, aided in their contraction (1) by the

found upon each muscle fiber. These terminals and the muscle fibers innervated by them are grouped as units. Such a unit is made up of the total number of muscle fibers innervated by the terminal branchings of one motor cell body within the central nervous system. No one knows whether this unit of muscle fibers is contained within one single muscle bundle or distributed among several anatomically discrete bundles. The control of the use of skeletal muscle is mediated, therefore, by way of single terminals upon individual muscle fibers, acting as a group.

The control of the use of this musculature presents many facets. Some of these facets are generalized and characterize a genus; others are more specialized and distinguish species and subspecies. The domestic cat uses its musculature very similarly to that of other members of the feline family. That use, although comparable, is different from that made by dogs. Great variation delineates the various members of the primate family. Consequently, the results of studies of the cat or the dog cannot be transferred to the rabbit or to the monkey. And certainly although suggestive, the analysis of the control of skeletal musculature characteristic of the macaque cannot be transferred to man.

The primate shares with all other forms the maintenance of posture and necessity for progession. The primate is differentiated from other mammals by the ability to use the upper extremity for the manipulation of objects; and man from other primates by his bipedal posture and bipedal progression. In man, the upright position is maintained not by an excess of tone in the extensors but rather by a nice balance between contraction of extensor and flexor sheets in the lower extremity—with a certain emphasis on the isotonic contraction of the anatomical flexors. There is fixation at both the pectoral and pelvic girdles, together with contraction of the M. rectus abdominalis. Bipedal progression is in one sense quadrupedal, for as one leg is protracted, the opposite arm is also protracted. Subsequent retraction of the lower extremity is accompanied by retraction of the contralateral upper extremity. Again, the girdles are fixed so that they do not twist on the trunk. The young monkey (3 months to 1 year of age) frequently uses bipedal progression. He fixes the pectoral girdle either by elevation of his arms or by the maintenance of simultaneous protraction. In these positions, the upper extremity fails to mimic quadrupedal progression. After a year of age, the macaque rarely progresses except in the usual quadrupedal manner.

ent peripheral nerve fibers carrying different varieties of sensory impulses. Other groups of cells occur, designated as adjustor nuclei, and are characteristic of several segments of the spinal cord. Each dorsal root contains nerve fibers which innervate sensory endings in the skin and subcutaneous connective tissue as well as those found in striated muscles; in some dorsal roots (thoracic and upper lumbar), visceral afferents are added. Each ventral root contains large efferent nerve fibers (α fibers) innervating skeletal muscles and small nerve fibers (γ fibers) terminating upon the intrafusal fibers of muscle spindles. To these may be added in some ventral roots the visceral efferents of the autonomic nervous system.

The control of the use of skeletal muscles of the body, as opposed to those of the head, is exercised through arrangements of neurons found within the central grey matter of the spinal cord. These arrangements of neurons, which form one of the fundamental physiological mechanisms, are designated by the term reciprocal innervation.

Sherrington's reciprocal innervation has been given a new form by the results of intracellular recording of postsynaptic potentials by Eccles and his collaborators (Eccles, 1957). A single nerve fiber from sensory endings on muscle spindles divides within the central grey matter, sending a lateral branch to synapse with the motor cells innervating the M. quadriceps femoris and a medial branch to synapse with cells which Eccles believes to lie in Cajal's (1909) intermediate nucleus. The axons of the cells of this nucleus which lie in this and in more caudal levels synapse with motor cells which innervate the M. biceps semimembranosus. The monosynaptic pathway is interpreted as excitatory; the disynaptic, as inhibitory. The central distribution of the nerve fibers from Golgi endings on tendons and on the insertions of extrafusal muscle fibers differ from that of the sensory ending of the muscle spindle by the intercalation of a neuron in the former pathway.

These two proprioceptive endings have recently exchanged their previously allotted functions. The Golgi endings present a high threshold to stretch and their discharge increases during active contraction (Hunt and Kuffler, 1951b). On the other hand, the spindle afferents have a low threshold to stretch and their discharge rate decreases or ceases during active contraction. The sensory endings upon the intrafusal fibers are activated by contraction, elicited by stimulation of their polar or motor endings via small nerve fibers which leave the ventral root with the larger nerve fibers destined for the extrafusal muscle fibers

synergists or cooperating muscle groups and (2) by the antagonists (which in this type of activity are more frequently slightly contracted than completely relaxed) as well as (3) by the fixation of more proximally lying muscle groups. On the other hand, the contraction of the musculature of the cooperating extremity may move down the arm involving in its passage either extensor or flexor sheets of muscle. These synergistic activities may be stopped, reversed, or fixed in any stage of their activity, receiving either an increment or decrement of tone.

How does the central nervous system manage to accomplish this beautiful use of skeletal muscle in the primate?

Because all experimental data derived from man is dependent upon chance vascular accidents or upon the intervention of neural surgery, it is necessary to utilize such primates as the macaque (the apes are too expensive) to analyze the relation of different fiber tracts to the use of skeletal musculature by the primate. Nonetheless, when the results of comparable lesions are considered, those which follow experimental ablations of the central nervous system of the *Macaca mulatta* suggest a similar relationship of results to site of injury as in man. Besides the method of ablation, which allocates to the part removed the loss apparent in the resulting change in the use of the primate's muscles, there are two other methods. Surfaces of the cortex cerebri or cerebelli can be stimulated under anesthesia in the macaque, without anesthesia in man. Deep structures in animals can be stimulated and the position of the buried electrode subsequently determined. Electrical recordings may be made from the surface of the cortices or from deeper structures. Our understanding of the functional contribution of different parts of the central nervous system to the control of the use of skeletal muscle in animals is dependent upon our interpretation of the results of these methods of study.

III. SEGMENTAL ORGANIZATION

Segmental organization of the central nervous system is more theoretical than real; this is so because organization of the spinal cord into levels or segments is superimposed upon the medulla spinalis by the enveloping bony covering of the vertebra. No single segment or level is able to function alone. Skeletal muscles are innervated both afferently and efferently by spinal roots from several levels. Within the central grey matter, motor cells are grouped as nuclei which innervate particular muscles and sensory cells are grouped as nuclei related to differ-

extremity on the operated side, particularly in those of the extensors of the knee, the adductors of the thigh, the ventroflexors and the invertors of the foot. The tendon reflexes of the M. quadriceps femoris, of the hamstrings and of the ventroflexors of the ankle, as well as of the invertors of the foot, are brisk. The inhibition and facilitation evoked by removal of the activity of these dorsal roots are of tone, not of movement. As phenomena, they are confined to the lower extremity, and exaggerated in both of these extremities whenever these particular roots are severed bilaterally (Hines and Knowlton, 1952).

This inhibition has no relation to the inhibition of reciprocal innervation, as studied by Eccles and his collaborators (1957), for that inhibition is restricted to that of the homolateral opposing flexors. The site in the spinal cord of the inhibitory nuclei which change the polarization of motor cells in so many nuclei of the ventral horn is as unknown as that of its counterpart, the site of facilitatory nuclei. They may not exist. The intermediate nucleus selected by Eccles as an inhibitory nucleus is probably not the one concerned because the increment in tone is found in the flexors as well as in the extensors of the knee. There is, in the macaque, at all levels of the spinal cord in which Clark's nucleus is not found, a discrete group of large nerve cells in the dorsomedial part of the central grey matter.

IV. LONGITUDINAL ORGANIZATION

A. Contribution of Sensory Systems

Each sensory system in the central nervous system contributes to the use of skeletal muscle. Skilled movements are directed by the eyes and by the ears as well as by proprioceptive systems. General cutaneous sensibility plays an appreciable rôle in some motor adjustments; whereas, it is difficult to discover the rôle of the chemical senses of taste and smell. Indeed, sight can direct motor performance rather adequately.

The neuron pathways which ascend for short distances within the spinal cord and form with short descending systems the fasciculus proprius are able to maintain even in spinal man multiple reflex activity (Kuhn, 1950) Although tone waxes and wanes in the extensors of the lower extremity of these injured men, the upright posture of standing can in some individuals be assumed with help, but not maintained. The adjustments between flexors and extensors of the lower extremities and

(cat; Hunt and Kuffler, 1951a). This arrangement allows the activation of the sensory ending of the spindle to be controlled centrally as well as independently of the isotonic contraction of extrafusal muscle fibers. The "small nerve reflex" can be regulated not only by stretch upon the muscle itself (Hunt, 1952) but also by stimulation of touch, pressure, and pain receptors in the skin and subcutaneous connective tissue (Hunt, 1951).

The interaction between these two proprioceptive organs is able to produce some regulation between antagonistic muscles of the same level. For, as the tension rises within a muscle in the process of contraction, the activity of the tendon endings increases and the spindle activity decreases. The motor neurons which by chance have not been activated become less excitable. The monosynaptic response from the spindle is inhibited and the antagonist response is facilitated. The opposing muscle is gradually stretched; the spindle afferents then excite the motor neurons to that muscle, the synergists are facilitated and the antagonists are inhibited.

These studies have implicated (Eccles, 1957) the intermediate nucleus of Cajal as the inhibitory nucleus in single reciprocal innervation and suggest that the maintenance of tone, assigned for years to nerve impulses entering the spinal cord via the dorsal root, is secondary to the excitation of the cell bodies (as yet unidentified) of the small nerve fibers innervating the polar ending on intrafusal muscle fibers.

Ever since Brongeest found that the skeletal muscles of the hind legs of a frog lost their tone after the dorsal roots of the lumbosacral plexus were cut and Sherrington's follow up with deafferentation of the muscles of the upper extremity of a monkey, tone in a particular skeletal muscle has been considered to be maintained at the segmental level by the dorsal roots which supply it. If, however, the dorsal roots of L, to L, which supply the sensory innervation to the M. quadriceps femoris of the macaque are severed, the palpable loss of tone will not be as great as if the dorsal roots of L_6 and L_7, are severed. Indeed, surgical division of these two roots is followed by a great decrement of tone in the whole extremity on the side of the lesion. The dorsal root of L_6 contains more of these facilitatory nerve fibers than does that of L_7 (the total number of nerve fibers is also much greater). Bilateral division augment this condition to such an extent that the extremities hang loosely when the monkey sits in the examining chair. On the other hand, division of the dorsal roots of S_1 and S_2 increases tone in all the muscle of the lower

neurons in the nucleus cuneatus, with a synaptic delay of only 0.6 msec. (Therman, 1941). Anatomically, the axons of these neurons and those of the nucleus gracilis have two destinations, the cortex of the cerebellum and the cortex of the parietal lobe via the medial lemniscus and the nucleus ventralis posterior lateralis. In the cat, axons of cells which lie within these two nuclei ascend among the descending fibers of the pyramids and terminate in the motor and sensory cortices without a synapse in any thalamic nucleus (Brodal and Wahlberg, 1952), a spinocortical tract.

The proprioceptive organ of the head is the labyrinth. The labyrinth presents two types of endings, one, the crista, capped by the cupola, found in the ampullae of the semicircular canals, and the other, the macula, covered by an otolith, located within the utriculus and the sacculus. The function of the sacculus has resisted all attempts at analysis. The cristae are sensitive to increments in angular acceleration; there is one for each plane in space. The motor adjustments to these displacements are compensatory. These compensatory movements involve the extrinsic muscles of the eyes and the muscles of the trunk. The muscles of the trunk respond, particularly when the crista of the horizontal canal is stimulated. The eye muscles are related via the fasciculus longitudinalis medialis to each of the three canals. The lateral and medial recti deviate the eyes opposite to the direction of the rotation in the horizontal plane; the dorsal and ventral recti, to that of the anterior canal; the superior and inferior oblique, to that of the posterior canal. The utriculus is stimulated maximally only when the otolith hangs from the macula and minimally when it presses upon the macula (Magnus and de Kleijn; see Magnus, 1924). This is the organ which effects tone in skeletal muscle. In the position of maximal stimulation, after the dorsal roots of C_1 to C_3 have been cut, the decerebrate animal shows maximal extensor tone (Magnus, 1922).

Although the exteroceptive sensory systems may direct the use of skeletal muscle in skilled performance, they seem to have little to do with the maintenance of tone or the interpretation of position in space. Rather, the evidence presented suggests that the proprioceptive systems are those which aid in the inhibition or in the facilitation of tone in skeletal muscle. Also, at the spinal level, the proprioceptive afferent by its central connections facilitates contraction of extensors and inhibits that of the opposing flexors and the reverse (Granit, 1956). The remarkable excitatory action, recently discovered by electrical stimu-

the fixation of the trunk and girdles necessary for the maintenance of standing are lacking.

The ascending systems within the spinal cord fall naturally into two groups, exteroceptive and proprioceptive. The exteroceptive group include the spinothalamic tracts and the spinotectal tracts carrying general cutaneous sensations to the thalamus and midbrain, respectively. The four proprioceptive systems are the two spinocerebellar tracts (dorsal and ventral) and the fasciculi gracilis et cuneatus. A transient diminution of tone in the ipsilateral muscles below the lesion follows section of the dorsal spinocerebellar tract (Ferraro and Barrara, 1935). No report has been made of the results of a similar lesion in the corresponding ventral tract. The ataxia of syphilis has been assigned to degeneration of the dorsal columns. Similarly, Foerster (1936) described ataxia and the loss of the sense of position and passive movement on the side below a stab wound which severed the dorsal funiculus.

The nerve fibers of the dorsal spinocerebellar tract, activated by impulses from muscle spindles, and entering the dorsal root via Group Ia and Group II fibers (Laporte *et al.*, 1956), synapse on the giant nerve cells of Clark's Column (Szentagothai and Albert, 1955) forming large and small terminals. The area of contact of each large terminal extends for several hundred square microns; that of smaller ones is not larger than the ordinary boutons terminaux. Group Ia and Group II nerve fibers furnish the giant terminals. Some of the smaller synaptic terminals may be axons of nerve cells which lie in the dorsal horn, others may be collaterals of spinocerebellar fibers. The same tract fiber can be activated by afferent volleys given different muscle nerves. Consequently, the synaptic relay could possess a function of coordination (Laporte *et al.*, 1956). No study has been reported which can correlate nerve endings in the periphery with degenerating boutons on the scattered cells of Stilling. Since the ventral spinocerebellar tract (Foerster and Gagel, 1932) receives additions at all levels of the spinal cord, it may play a significant rôle in control of skeletal muscles of the extremities.

The dorsal root fibers which enter the dorsal columns to form the fasciculus gracilis and the fasciculus cuneatus belong to the large group of nerve fibers within the dorsal root. Peripherally, these nerve fibers innervate muscle spindles and Golgi-Mazzoni tendon terminals. Single impulses experimentally evoked in the nerve fibers of the dorsal column of the fasciculus cuneatus have a remarkable excitatory effect on the

projection, those of association, and those which are recruiting. The first two systems form looped circuits with the afferent fibers to the cortex, the latter via the nuclei of association, reverberating circuits which produce asynchronous cortical rhythms (Starzl and Whitlock, 1952). Before the intensive use of electrical devices for stimulation and recording nervous activity, the function of the efferents from the sensory cortices to the thalamic nuclei were interpreted by Head as inhibitory.

Besides these corticothalamic systems which originate in layer VI (Winkler, 1929), there are others. Many corticifugal systems share common destinations. The superior colliculus receives axons from the frontal lobe (area 4, Mettler, 1935), the parietal lobe (area 7, Peele, 1942), and the occipital lobe (areas 18 and 19, Mettler, 1935; Lemmen, 1951; area 22, Lemmen, 1951). The inferior colliculus, moreover, restricts its cortical axons to the temporal lobe, whereas the pretectal area receives fibers from the parietal lobe (areas 5 and 7, Peele, 1942). The nucleus ruber entertains fibers from the frontal lobe areas 4, 6, and 4s (Mettler, 1948a; the present author found none from the anterior division of 4, Hines, 1943), from the postcentral gyrus (Mettler, 1935; Lemmen, 1951), and from area 19 of the occipital lobe (Lemmen, 1951). The subthalamus receives efferent fibers from the area frontalis agranularis (Levin, 1936; Verhaart and Kennard, 1940; Hines, 1943). The substantia nigra has a broad spectrum of corticifugal systems originating from the area frontalis agranularis and area 8 of the frontal lobe (Levin, 1936; Verhaart and Kennard, 1940; Hines, 1943), from the superior gyrus of the temporal lobe (area 22, Mettler, 1936; Bucy and Klüver, 1940), from areas 3 and 5 of the parietal lobe (Peele, 1942), and perhaps from area 19 of the occipital lobe (Lemmen, 1951).

C. The Core of the Brain Stem

At the present time, the control of skeletal muscle cannot be discussed without a critical appraisal of the function of the tegmentum and reticular formation of the medulla oblongata. This great core of the brain stem which defied analysis for so many years has begun to yield some factual material to the inquisitive deep recording and stimulating electrodes. The interruption of the continuity of these fibers and their cell bodies either in the region of the hypothalamus (Ranson, 1939) or cephalad to the level of the superior colliculus (Peterson *et al.*, 1949) produced a monkey distinguished by loss of motor initiation, a

lation and recording of single nerve fibers in the dorsal spinocerebellar tract and in the fasciculus cuneatus, suggests that stimulation of only a few muscle afferents are necessary to produce this result.

B. Contributions by Descending Systems

The descending systems which control the use of skeletal muscle in the primate fall naturally into two groups, pyramidal and extrapyramidal, because of their anatomical positions within the medulla oblongata. The pyramidal group are long descending systems of nerve fibers which, passing through the brain stem into the medulla oblongata and lying in the pyramids, do not synapse until they terminate within the central grey matter of the spinal cord.

The pyramids of the monkey and of man contain two descending systems, (a) *the old corticospinal tract* which stems from the giant cells of Betz and other large pyramidal cells in layer III (Spielmeyer, 1906) and in layer V of area 4 (Wohlfahrt, 1932), and from area 6, (Minckler *et al.*, 1944), degeneration after removal of area 6 in man; Woolsey and Chang (1948), evidence, that of antidromic evoked potentials; Levin (1936) *found no degenerated myelin in the pyramids* after ablation of area 6, and (b) *the new corticospinal tract*, the cells of origin of which are the large pyramidal cells in layer III of area 1 (Levin and Bradford, 1937) as well as from pyramidal cells in layer III and layer V of the remaining parietal lobe (Levin and Bradford (1937) retrograde chromatolysis, lesion in position of corticospinal tract at C_4). Peele (1942, Macaque, Marchi method) found degenerating myelin in the ipsilateral pyramid, in the lateral corticospinal tract as low as the lumbar levels, contralateral to lesions in the postcentral gyrus and in area 5; whereas, lesions in area 7 extended only to the cervical levels of the ipsilateral side. For the sake of clarity, the old corticospinal tract will be referred to as the frontospinal and the new, as the parietospinal (Wagley, 1945). Besides the two corticospinal systems, Wahlberg and Brodal (1953) find degenerating axis cylinders among the sound nerve fibers of the pyramids, of the ventral and lateral corticospinal tract in the spinal cord of the cat, after removal either of the occipital lobe or of the temporal lobe.

The extrapyramidal systems fall naturally into two categories: (1) those which terminate in the dorsal sensory nuclei of the thalamus and (2) those which end in the nuclei of the brain stem. The thalamic nuclei which receive such corticifugal systems are namely, those of sensory

projection, those of association, and those which are recruiting. The first two systems form looped circuits with the afferent fibers to the cortex, the latter via the nuclei of association, reverberating circuits which produce asynchronous cortical rhythms (Starzl and Whitlock, 1952). Before the intensive use of electrical devices for stimulation and recording nervous activity, the function of the efferents from the sensory cortices to the thalamic nuclei were interpreted by Head as inhibitory.

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masklike face, and a loss of the desire to eat. The long ascending sensory systems were intact and the corticospinal tracts were uninjured. Besides this "loss of the will to move," these animals slept (Bremer, 1935).

The reticular system contains both ascending and descending systems. The descending systems stem from the corpus striatum, the hypothalamus, the midbrain tegmentum, and even from the cerebral cortex. The ascending systems within this complex region arise from reticular nuclei, from the spinal cord, or are collaterals of ascending sensory systems. Not only do collaterals from each of the four lemniscus systems enter the reticular formation, a recent rediscovery of Winkler's (1929) findings, but also axons from the nucleus fasciculus solitarius (Allen, 1923), from the posterior accessory optic tract (fibers not medullated, either in man, Marburg, 1903; or in the monkey, Gillilan, 1941), from the ascending visceral sensory systems, as well as from the vestibular system (Gerebtzoff, 1940) enter this region. Thus the reticular system receives impulses from each kind of sensory system found in the mammalian body. Nonetheless, some organization is emerging. The Scheibels (Scheibel *et al.*, 1955) find that convergence of afferent impulses on units of the reticular formation, although widespread, is not unlimited. Indeed, convergence patterns on individual units vary greatly.

The function of ascending systems in the reticular formation may not be restricted to keeping the cerebral cortex awake by way of recruiting thalamic nuclei (Starzl and Whitlock, 1952). There are direct spinoreticular systems which can be followed in the degenerated state as far cephalward as the level of the abducens nucleus (Morin *et al.*, 1951). Some of these fibers synapse in the lateral reticular nucleus of the medulla and terminate medially and laterally in the midbrain tegmentum. The peripheral fibers innervate joints and tendons. The nucleus of origin is anatomically unknown (cat; Morin, 1953).

Throughout the reticular formation, many anatomically discrete groups of nerve cells exist. At the level of the nuclei of each cranial nerve in man, the reticular formation presents specialized nuclei, some of which are medially placed, others laterally. Brodal (1956) finds that the medial two-thirds of the reticular formation contain the cells of origin of the long ascending and of the long descending tracts of this region. Into this region, many corticoreticular fibers terminate (from motor cortex and area 24, cat, Rossi and Brodal, 1955); and from this

region some of the reticulospinal fibers originate, coinciding with the inhibitory area (Magoun and Rhines, 1946; Niemer and Magoun, 1947). The site of the cells of origin of these reticulospinal systems found rostrally in the pons and midbrain is less extensive than that of the facilitatory region of Magoun and his associates. In other words, the inhibitory impulses reaching the spinal cord arise in the medial part of the posterior division of the reticular formation (Magoun and Rhines, 1947; Ward, 1947), whereas the facilitatory impulses stem from cells located more rostrally, both laterally and medially in the tegmentum. This facilitatory system receives contributions from the basal part of the diencephalon, from the corpus striatum (globus pallidus in particular; Mettler, 1941, 1943, 1944), and perhaps from the nuclei of the thalamus. Furthermore, it is augmented by fibers from the midbrain and the rostral part of the medulla oblongata. The reticulospinal tracts carrying facilitation lie throughout the lateral funiculus, concentrated near its lateral border (Niemer and Magoun, 1947). The reticulospinal tracts carrying inhibitory impulses are found in the ventral funiculus and in the ventral part of the lateral funiculus (Niemer and Magoun, 1947; Wagley, 1945). Unilateral lesions in these regions of the spinal cord (Wagley, 1945) show bilateral chromatolysis in the nerve cells of the nucleus reticularis lateralis in the midbrain. Other origins of these inhibitors are found in the caudal reticular formation, for Bodian(1946) found many large degenerated cells in this region during the spastic stage of experimental poliomyelitis in the monkey. The inhibitory fibers may terminate on adjustor neurons in the intermediate area of the spinal cord grey (cat; van Harreveld and Marmost, 1939). Electrophysiological analysis of the results which follow or accompany the activation of descending facilitatory systems (i.e. the vestibulospinal and reticulospinal, cat) led Lloyd (1941) to conclude that these fibers terminate directly upon the ventral horn cells and upon neighboring nerve cells. The indirect pathway implicated nearby neurons in such a way that the facilitation of the ventral horn cell was augmented and its duration prolonged.

Within the generalized reticular-tegmental region, three specialized nuclei have developed—the nucleus ruber, the substantia nigra, and the subthalamus. The nucleus ruber projects its axons cephalward to the globus pallidus, to the arcuate and ventrolateral nuclei of the thalamus, and to the subthalamus, and caudalward to the spinal cord (monkey; Carpenter, 1956). Injury to this nucleus alone is followed by

a great reluctance to move, without any change in the use of skeletal musculature. The hypokinesis which followed brain stem lesions reported above must have included division of the corticorubral tracts. Certainly, the lesion in the spinal cord, which resulted in an unwillingness to use the muscles on the side of the lesion, severed the rubrospinal tract as well as reticulospinal pathways (Wagley, 1945). Unilateral lesion of the corresponding brachium conjunctivum plus either unilateral injury to the nucleus ruber (Orioli and Mettler, 1957) or a lesion in the ipsilateral substantia nigra (Carrea and Mettler, 1955) adds only hypokinesis to the pre-existing results. The substantia nigra receives a wealth of corticifugal fibers, reserves a special site for their reception, according to cortical origin, and has not as yet divulged its function even to electrolytic lesions (Ranson, see Cannon *et al.*, 1944). Stereotaxtic bilateral injury to the subthalamus in the macaque (Carpenter *et al.*, 1950) is followed by bilateral choreoid hyperkinesis, which can be eliminated or decreased by an 8% reduction of the nervous tissue within the globus pallidus (bilaterally).

The cerebellum, related on the afferent side to the exteroceptors and proprioceptors of the spinal cord, to nuclei in the reticular formation, to the tectum of the midbrain as well as to the motor cortex, the parietal lobe, the occipital and the temporal lobes, projects upon the reticular formation via pathways which originate in one or more of the three cerebellar nuclei—the medial or fastigial, the intermediate or globosus and emboliformis, and the lateral or dentatus. The bulk of the projection from the nucleus dentatus as the crossed ascending limb passes into the reticular formation, transverses the nucleus ruber (the uncrossed ascending limb bypasses this nucleus) to end in the ventrolateral nucleus of the thalamus and in the globus pallidus. The descending limb has three components: (1) to three discrete nuclei in the tegmentum of the pons, (2) entering the medial longitudinal fasciculus, and (3) to the cervical spinal cord (Carrea and Mettler, 1954). Cerebellar ataxia was found to result from injury either to the dorsal or intermediate components of the brachium conjunctivum destined for the large celled nucleus ruber or to the descending limb of the superior brachium. Cerebellar tremor followed lesion of the component of brachium conjunctivum destined for suprasegmental levels, i.e. of the nucleus dentatus, of the midbrain tegmentum (Carrea and Mettler, 1955), and of the nucleus ventrolateralis of the thalamus (Walker, 1938). Hypotonia was not mentioned in either of these two studies. Botterell and Fulton

(1938) described hypotonia after either removal of the lateral hemispheres or injury to the nucleus dentatus. To the hypotonia which appeared after the first lesion, tremor and ataxia were added after the second. The latter finding agrees with the work of Carrea and Mettler.

The fastigial nucleus is credited with ability to inhibit extensor tone, because Sherrington found that electrical stimulation of the anterior lobe decreased extensor tone of decerebration (cat) and that removal increased decerebrate tone without change in its distribution. Repetition of these experiments have given similar results. The decerebrate state can be produced by removal of the inhibitory activity of the reticular formation by lesions in the midline of the medulla and allowing the facilitatory mechanism to be stimulated by afferents from sensory systems of the spinal cord and medulla oblongata (macaque; Ward, 1947). Facilitatory activity, on the other hand, can be destroyed by lesions in the middle third of the reticular system in the midbrain or by lateral lesions in more caudal levels. Apparently, in the primate the vestibulospinal tract cannot without the reticulospinal fibers in the ventral division of the spinal cord maintain the hypertonicity of the decerebrate state (Bach and Magoun, 1947).

In the analysis of this state in cats, Sprague and Chambers (1953) describe collapse of decerebrate rigidity as following destruction of the nucleus fastigii and of Deiters' nucleus. The pontobulbar reticular formation remained intact. Apparently, loss of Deiters' nucleus and consequently the majority of the facilitatory activity of the vestibulospinal tract left the reticular formation without adequate afferent stimulation. The results given suggest that the impulses discharged by the fastigial nucleus into the reticular formation are dominantly facilitatory, not inhibitory—an apparent contradiction to the results of Sherrington's original experiments. Or does destruction of these two nuclei release the crossed inhibitors of this hypertonic state?

The activity of the crossed inhibitors of extensor rigidity can be seen in a decerebrate cat in which the right fore leg has become atonic after removal of the caudal pole of the opposite nucleus fastigii. This crossed inhibition of extensor rigidity when removed (Moruzzi and Pompeiano, 1957) by deafferentation of the spastic left leg, by cutting the left VIIIth nerve or the left vestibular nerve, or by hemisection of the spinal cord on the left side between T_{12} and L_1, the atonic right foreleg becomes hypertonic and the left hypertonic foreleg becomes atonic. Apparently the crossed afferents are inhibitory, the ipsilateral af-

ferents, facilitatory. Myotatic influences can be removed by cutting the dorsal roots to both forelegs. Both forelegs become atonic; the hind legs, tonic. This local modification of the decerebrate state by local removal of the activity of several varieties of afferent inhibitors or facilitators suggests that local inhibitors are able to take precedence over general arousal of the more cephalic facilitators, at least in the cat.

The brain stem which contains the nervous mechanisms which assure smooth movement and facilitate and inhibit tone or movement also contains complex nerve mechanisms which when stimulated directly produce complex movements, such as flexion of one extremity, extension of the opposite one, with either extension or flexion of a third. Eyes, ears, and facial muscles were sometimes included. Crosby (1956) explored the tegmentum of the macaque under anesthesia. The movements elicited resembled those which she and her associates had evoked by stimulation of the cortices of the parietal and temporal lobes. She thought that the impulses so aroused traveled to motor centers of the brain stem and spinal cord via multineuron arcs. These were identified as corticostriatal (Dusser de Barrene *et al.*, 1940), striatotegmental, or striatorubral, thence to the spinal motor cells via the rubrospinal or tegmentospinal systems.

To Sprague and Chambers (1954) no general facilitators or inhibitors of tone exist, because they have been unable to duplicate these painstaking experiments of Magoun and his co-workers, by stimulating the reticular formation in unanesthesized cats. Direct stimulation with implanted electrodes placed medially elicited flexion of the ipsilateral legs and extension of the contralateral legs plus the inhibition appropriate to reciprocal innervation. With the electrodes placed laterally, the pattern of tonic flexion and extension was completely reversed. When the intensity of the stimulating current was reduced, the cat circled and came to rest in the normal curled position of sleep.

These interrelated neurons form the substrata in the brain stem and spinal cord which the cortex cerebri utilizes in its control of skeletal muscle. These brain mechanisms adjust the tone in skeletal muscle to fit the task and lend to all movement the smoothness and sequence of contraction that is termed normal.

Here also is found the basis for organized movements such as quadrupedal progression and the mass organization of synergistic activities.

V. THE MOTOR CORTEX

The majority of investigators have emphasized the lack of correspondence between contraction of skeletal muscle elicited by stimulation of the precentral gyrus and the use of that musculature made by the animal or by man. This discrepancy has been noted particularly by neural surgeons who have stimulated the cerebral cortex of conscious man prior to removal of diseased tissue. Topical localization of contraction of single muscles or parts of muscles and low intensity of the stimulating current distinguish the precentral gyrus of all primates.

On the other hand, many movements evoked by the sine wave current used on the precentral gyrus of fetal and infant macaques present a striking similarity to those in use by the young monkey at the time of stimulation. Because Hines and Boynton (1940) could not assign these movements to the extrapyramidal or to the corticospinal systems, they were classified respectively as holokinetic and idiokinetic. The holokinetic movements were those of infantile behavior patterns, patterns of progression, movements of girdle and neck musculature, as well as rhythmical movements of lip, tongue, and upper extremity. In early postnatal development, the idiokinetic movements lacked discreteness. Rather, they were co-innervations resembling those used by the conscious infant at the age they were elicited. Strangely enough, during the last month of gestation and the first two weeks of postnatal life, the cortical electrode was able to elicit such discrete movements as extension of the thumb or flexion of the hallux, which the monkey did not make at the age of stimulation; but, thereafter, the evoked contraction corresponded extraordinarily well with those used by the infant itself.

The recent explorations of the cortex cerebri of monkeys and of men are concerned with elicitation of contraction of skeletal muscle rather than with inhibition or facilitation of its tone. The threshold for excitability of the cortical points in these new motor fields is higher than that of the "motor" cortex in the precentral gyrus of primates and the movements obtained are far more complex. Each of these fields is independent of the "motor" cortex and its corticifugal systems.

A. MOTOR AREA II

The oldest of these recently discovered motor fields (Sugar *et al.*, 1948) joins the most ventral part of the precentral gyrus in the monkey's

cortex, follows the posterior frontal operculum, and spreads out over the insula and under the anterior parietal operculum (Fig. 1). The stimulating voltage was extremely high (10 to 20 v.), twice as much as that necessary in these monkeys to evoke movement of the thumb by stimulating the precentral motor cortex. Either the type of stimulating current was not adapted for excitation of these cortical areas (including the precentral gyrus), or the resistance of the cortex was very high. Somatotopical localization was ragged. The arm area overlapped that

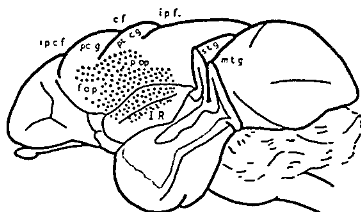


FIG. 1. Line drawing of the brain of a macaque. The superior and middle temporal gyri and part of the inferior temporal gyrus have been removed, so that the parietal (pt. op.) and frontal (f. op.) opercula may be seen. The medial surface of the Island of Reil (I.R.) has been pushed up into view. Other abbreviations are: pc. g., precentral gyrus; pt. cg., postcentral gyrus; c.f., central fissure; and i.p.f., intraparietal fissure. The stippled area outlines the limit of motor cortex II as described by Sugar *et al.* (1948).

of the face dorsally, and that of the leg ventrally. Movements of the thumb and arrest of respiration were generously and without apparent organization peppered over the insula. Posteriorly, this motor cortex overlapped the second somatic area (Woolsey and Fairman, 1946).

B. THE SUPPLEMENTARY MOTOR CORTEX

The second of these motor fields was first discovered in conscious man by Penfield and Welch (1951) on the medial surface of the superior frontal gyrus and was named the supplementary motor cortex. The region outlined by these authors is extensive, stretching from the leg area in the paracentral lobule to the anterior border of the medial subdivision of Brodmann's area 6. Penfield and Welch are particularly interested in discovering new sites which give inhibition of speech. They uncovered no somatotopical localization on the medial surface for leg,

arm, or face. The movements elicited were complex, frequently located ipsilaterally, and the intensity of the stimulating current was greater than the threshold values of excitable loci on the precentral gyrus. Erickson and Woolsey (1951) substantiated the loci but found evidence of some somatotopical localization.

Before this study was published, Bates (1953) had seized the opportunity of stimulating the medial surface of a sound hemisphere, exposed by removal of the opposite cortex cerebri because of infantile hemi-

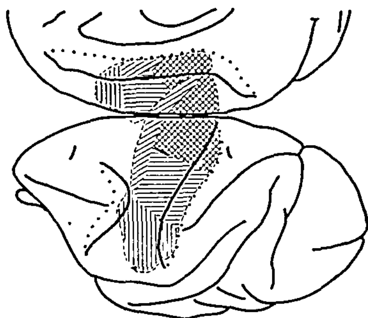


FIG. 2. A diagram of the lateral surface and adjoining medial surface of the precentral motor cortex of the macaque's brain. The fissures are drawn as if they were open, in order to reveal the extent of the motor areas therein. The precentral motor cortex occupies the precentral gyrus, the anterior bank of the central fissure, a small area in the posterior bank of the central fissure, a small area in the posterior bank of the inferior precentral fissure, as well as the posterior part of the medial surface of the superior frontal gyrus. The supplementary motor cortex occupies the medial surface on the superior frontal gyrus anterior to the leg area of Brodmann's 4, as well as the dorsal bank of the sulcus cinguli. The anterior limit on the medial surface stops short of the anterior boundary of the homologous area discovered in Cercopithecus by Horsley and Schafer (1888). (Redrawn from a figure made by Woolsey and used by Erickson and Woolsey in a paper presented before the American Neurological Association in 1951.)

plegia. The exploration, done under a general anesthesia after the ablation of the injured hemisphere, was confined to a small region about the medial tip of the central fissure. The resulting movements were complex and the threshold was high. Plotting the primary movements after the manner of Leyton and Sherrington (1917) showed some

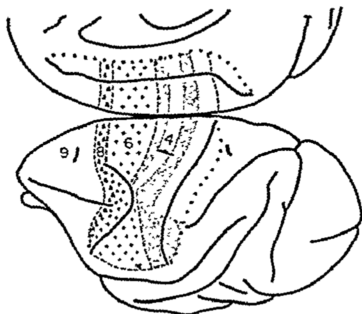


FIG. 3. This is Woolsey's (1951) diagram, upon which are projected the extent of Brodmann's areas (1903, 1906) as found by the author (unpublished). Comparisons of the extent of area 4 with that of the precentral motor cortex (FIG. 2) will reveal *that the latter extends more anteriorly than does area 4 on the lateral surface* of the cortex cerebri; while on the medial surface, the precentral motor cortex does not include the small region turned inward as the superior bank of the sulcus cinguli, cytoarchitectonically area 4. The supplementary motor cortex includes the dorsal bank of the gyrus cinguli in the "leg" area, the anterior border of area 4 on the medial surface, and all of the medial division of area 6, together with the dorsal bank of the gyrus cinguli. The extent of these differentiated cytoarchitectonic areas resemble those described for the cortical surface of the frontal lobe by Mettler (1948a).

Area 4 has been divided into four strips. The one which passes through the superior precentral fissure outlines the extent of the cortex, the removal of which is followed by some of the facets of the spastic state, namely, clonus, irriadiating brisk tendon reflexes, and the differential distribution of resistance to passive movement, in the contralateral extremities. The paralysis which persisted was confined to the abductors of the toes (Hines, 1936). The posterior clear area is the site of the lesion *which gives no permanent paralysis and little or no increase in resistance to passive* movement, no clonus, and no brisk tendon reflexes Initially, there is a real paralysis in the limbs opposite the lesion and muscle tone is greatly decreased. This decrement in tone lasts for years. The paralysis is transient.

The cortex represented by the stippled areas were left intact, so that in the former operation, area 6 would not be injured and in the latter, the more anterior part of area 4 would be free from the trauma of the operation.

topical localization. Area 6 was not stimulated. In spite of a general anesthesia, Bates obtained contraction of ipsilateral muscles. One of these sequential complex movements stopped with ipsilateral extension of the ipsilateral hallux—a positive Babinski.

On the medial surface of the macaque's frontal lobe (Erickson and Woolsey, 1951; Woolsey *et al.*, 1952) confluent posteriorly with the precentral motor cortex and ending in the vicinity of the anterior border of area 6, lies the supplementary motor cortex. The two leg areas meet on the dorsal crest of the sulcus cinguli. The anesthesia was deep, the threshold was high, and the contractions of skeletal muscles, sequential and complex, were located without exception contralaterally (Fig. 2: compare with Fig. 3).

C. The Postcentral Motor Cortex

Again, stimulation of the postcentral gyrus with 60 c.p.s. sine wave current after both of these motor cortices were removed for weeks or months elicited a detailed representation of contralateral musculature which part for part followed the outline of tactile representation of the surface of the body (Woolsey *et al.*, 1942). The intensity of the stimulating current required to produce these movements was two to three times higher than that necessary to evoke contraction of muscle by stimulation of the precentral gyrus (Woolsey *et al.*, 1953). In man, however, Foerster (1936a) did not elicit movement by stimulation of the postcentral gyrus after the precentral gyrus had been removed.

D. Additional Motor Areas

Elizabeth Crosby and her associates called the electrically excitable areas found on the crest of the intraparietal fissure (Fleming and Crosby, 1955) and on the superior temporal gyrus (Schneider and Crosby, 1954) additional motor areas. The results of stimulation of the parietal lobe were an extension of Peele's report (1944). The majority of movements obtained on the parietal lobe was contralateral, while the majority of those evoked from the superior temporal gyrus were ipsilateral. The thresholds were high and varied between 2 and 14 v. Some somatotropic localization occurred. The movements obtained from these areas in the intact cortex were similar to those elicited subsequent to removal of area 4 or of area 4 and the postcentral gyrus. Nonetheless, the presence of these electrically active motor areas did not prevent the development of typical area 4 paralysis (Fig. 4).

Besides these four accessory motor fields, there are others first discovered by Ferrier (1876) and located by him within each lobe of the monkey's cerebral cortex. Ferrier's fields were subsequently lost because of Sherrington's pronouncement that complete flaccidity of skeletal muscle was the prerequisite for electrical stimulation of the motor cortex. Later, they were rediscovered by the Vogts (1926) for monkeys and by Foerster (1936a or b) for man.

Each lobe of the monkey's cerebral cortex presents its own motor

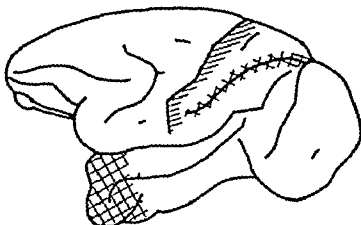


FIG. 4. Line drawing of a hemisphere of a macaque's brain upon which is projected the postcentral motor cortex of Woolsey *et al.* (1954) and the two additional motor cortices of Crosby and her associates. The "motor cortex" of the temporal lobe was discovered by Schneider and Crosby (1954); that of the parietal lobe, by Fleming and Crosby (1955). Peele's results (1944) of electrical stimulation of the parietal lobe are similar to those reported by the latter authors.

field, far from the origin of the frontospinal tract and electrically active even after the pyramids are surgically divided (Hines, 1943, 1947). Each of these motor fields is located within the cortical area to which thalamic nuclei of association project. Removal of any one of these fields except the one in the parietal lobe (part of area 7) does not visibly change the use of skeletal muscle by the macaque. After removal of area 7, the deficiency in motor performance is related to loss of recognition of objects by somaesthetic sensibility and to hypotonia of skeletal muscles of the upper extremity (Peele, 1944; Kennard and Kessler, 1940). Each of these fields responds to the stimulating current (60 c.p.s., sine wave) with adversive movements and quieting (Tower and Hines, unpublished; cf. Hines, 1947). The quieting effect causing cessation of spontaneous movement (the ether had been previously lightened) confers upon the macaque an appearance of attentive repose.

This effect is more easily obtained from the anterior field on the lateral surface of Brodmann's area 9 than from any one of the three posterior fields. The adversive movements common to these fields were conjugate devitation of the eyes plus at times that of the head as well. In the frontal field, these adversive movements sometimes included the axis, and the contralateral extremities, as well as the tail. Orientation of the ears was obtained from three of the four fields (not from the parietal lobe) (Fig. 5).

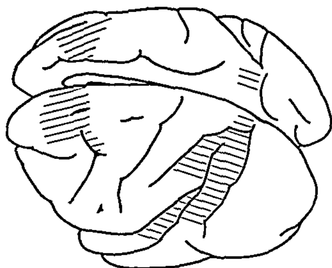


FIG. 5. Line drawing of the extrapyramidal motor areas of the four lobes of the cerebral cortex, as found in the macaque; used as a lantern slide to illustrate "The Motor Areas." These areas resemble those located by Ferrier (1876) outside of the precentral gyrus (Hines, 1947).

Three of these fields yielded other movements of the eyes, such as opening and dilation of the pupil from the frontal field (Smith, 1949), convergence from the parietal field (Tower and Hines, unpublished; cf. Hines, 1947), and closure of the eyes as well as constriction of the pupil from the occipital area (Walker and Weaver, 1940). Besides these movements of the eyes, the temporal and occipital fields responded with a complex movement called by Tower and Hines (Hines, 1947) a reaching and grasping act.

In the macaque, but not in man (Foerster, 1931), stimulation of area 17 oriented the eyes to the visual fields represented in the region stimulated (Walker and Weaver, 1940). Area 17 was at one time the only primary sensory projection area of the monkey's cortex which responded with contraction of skeletal muscle.

Outside of these regions, an electrode on the cortex around the caudal

end of the fissura principalis (area 8) evoked nystagmus and conjugate deviation of the eyes, whereas one on the cortex dorsal to that fissure elicited deviation of the eyes, either upward or downward.

These are the areas of cortical surface which have been excited by the parameters of the electric current now in use. The results of the early stimulations of the motor cortex have produced many of the interpretations of the fundamental relationships between nervous tissue and skeletal muscle. This multiplication of motor cortices challenges new interpretations; for with the exception of the supplementary motor cortex in the macaque, none of these motor areas are able to substitute for loss of the precentral motor cortex. This search for additional motor cortices is motivated by more than curiosity; for that search hopes to uncover some way of substituting the activity of these new motor areas for that of the precentral gyrus, which is sometimes congenitally and regrettably absent in man.

E. Participation of the Small Nerve Fiber in Control of Tone and Movement

Since decerebrate rigidity of the Sherringtonian type is ushered in by uniform rates of firing of small nerve fibers which innervate the intrafusal muscle fibers of the spindles, it was logical for Granit and his co-workers (Granit, 1956) to ask whether the activity of the ventral horn cells fired by the activity of the equatorial ending was organized for cooperation with direct excitation of the extrafusal fibers.

These workers found that the small nerve fiber of the spindle was facilitated or inhibited by the same cephalad areas of brain which had been found previously to be facilitatory or inhibitory of tone and of movement. Stimulation of the motor cortex of the cat (3 msec. shock) with a low strength of current elicited small nerve activity only; an increase in current strength added activity of the large nerve fibers to that of the small ones. Stimulation with needle electrodes in the lateral midbrain tegmentum found the large nerve fibers silent, while the rate of discharge in the small nerve fibers gradually rose from 8 to 40 per second and fired at maximum frequency for 20 sec. after the current had been removed. A similar stimulation of the pyramids selectively activated the spindle without either the slow recruitment or after-effect characteristic of facilitation of small nerve fibers which resulted from stimulation of the reticular formation. In some instances, spindle activity was excited by stimulation of the cerebellum.

Complete inhibition of both small and large nerve fiber discharge was obtained by stimulating regions from which inhibition of tone and/or of movement have been obtained by other methods. These regions were the medial reticular formation dorsal to the pyramids, the anterior part of the cingulate gyrus, and the anterior lobe of the cerebellum.

The "double reciprocal innervation" as a principle of reflex organization has not been found by Granit (1956) to be symmetrical in

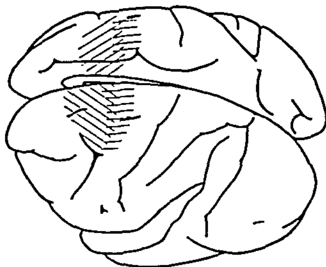


Fig. 6. [illegible] and grasping, inhibition of flexor tone and of the grasp reflex, when stimulated with the sine wave current (60 c.p.s.). With similar electrical stimulation, the posterior field (anterior 4 plus posterior boundary of 6) yields quadrupedal progression, extensor synergies, standing tone, and inhibition of extensor tone. These phenomena were elicited under light ether anesthesia not only before but also after surgical division of the pyramid ipsilateral to the cortex stimulated.

flexors and extensors. In the double sensory innervation of skeletal muscle, the afferents on the spindles facilitate the muscle which contains them and inhibits the antagonist; whereas, the Golgi tendon organs inhibit their muscle and facilitate their antagonist. There may sometimes be simultaneous contraction or simultaneous relaxation of both flexors and extensors.

Certain supraspinal lesions appear to shift this reflex organization. Reciprocal innervation which Hering and Sherrington (1897) obtained by stimulation of the precentral gyrus in monkeys disappears after the pyramids are severed (Tower and Hines, unpublished). Bosma and

Gellhorn (1946) showed that extensor and flexor muscles can be caused to contract simultaneously without an initial phase of inhibitory activity of the antagonistic muscle; and Chang *et al.* (1947) that contraction of single muscles can be elicited without concomitant inhibition of the opposing muscle or groups of muscles.

Cortical lesions upset the normal γ loop control of tone and movement in skeletal muscle so that reciprocal innervation seems to disappear. For example, reciprocal innervation disappears in states of tonic innervation of the flexors contralateral to removal of area 6 in the monkey and in tonic innervation of flexors and of extensors created by bilateral removal of area 6 and of the anterior border of area 4 (Hines, 1937, 1943).

Muscular tension stimulates the Golgi endings and they in turn inhibit the synergists. Is it the marked increase in tonic activity of the flexors of the fingers subsequent to bilateral removal of Brodmann's area 4 which causes the loss of dorsoflexion of the wrist when the fingers flex upon an object? Certainly in the stage of development of the infant macaque, known as tonic innervation, the flexors of the fingers are accompanied by flexion of the wrist (Hines, 1942). When the synergistic action of the dorsoflexors appears, tonic innervation had disappeared.

VI. POSTURE

The ability to maintain normal posture is the result of the sum of the activity of many parts of the nervous system. For example, the posture assumed by the infant macaque is related to the distribution of tone in his muscles and the mode of use of musculature which characterizes each stage in his postnatal development (Hines, 1942). Discrete organization of skeletal muscle demands fixation of the muscles of the girdles and trunk and of the more proximal ones of the extremity in order to free the distal ones for the initiation of movement.

A loss of fixation and a decrease in tone of the muscles follow removal of the cerebellar hemispheres in the macaque (never, however, as severe as in the baboon or in the chimpanzee; Botterell and Fulton, 1938). When the nucleus dentatus is included, tremor and ataxia are added (Carrea and Mettler, 1955).

Fixation is lost after bilateral surgical division of the pyramids (Tower, 1940). In standing, the legs are more adducted, the arms less so than in the intact animal. In sitting, the head falls forward (the extensors of the head are hypotonic), the back is extended, the arms

hang loosely in extension with a real droop of the shoulders, similar to the condition of the macaque in which the pes pedunculi was partially severed (Cannon *et al.*, 1944).

The hypotonia of total parietal lobe removal presents a posture similar to that assumed by the unilateral pyramidal animal (Peele, 1944). In the writer's experience, the degree of hypotonia seen in the macaque after simultaneous removal of the posterior division of Brodmann's area 4 and the postcentral gyrus is so great that no movement is initiated thereafter by the contralateral musculature (Hines, unpublished).

A differential distribution of increased tone also modifies posture. Bilateral removal of Brodmann's area 4 increased tone in the flexors of the trunk including the M. rectus femoris and decreased tone in the extensors of the back. Thus a sitting posture (Fig. 7) of flexion of the trunk (Hines, 1949) is very similar to that assumed by an adult macaque after bilateral removal of the precentral and the supplementary motor cortices (Travis and Woolsey, 1956). Additional loss of both parietal lobes reduces the monkey to a sitting posture characteristic of the 2-week-old infant macaque (Hines, 1942). Subsequent to bilateral removal of the frontal lobes (sparing both precentral face areas and the contiguus operculum), the monkey fixes his trunk by clutching a stationary object with one hand while he holds food with the other (Travis and Woolsey, 1956).

The associated tonic innervation of flexors and abductors together with the grasp reflex enables the infant monkey to maintain the posture of clinging to his mother either in the perpendicular or horizontal planes. Excessive tone in the extensors of the head places the macula of the utriculus in the position of maximum stimulation when the baby hangs on to his mother as she moves in the horizontal plane (Hines, 1942). Bilateral removal of the whole of Brodmann's area 6 plus that part of the gyrus cinguli which lies ventral to it causes a permanent bilateral grasp reflex (Richter and Hines, 1934). Removal of the supplementary motor cortex (Travis, 1955b) plus the precentral motor cortex, or bilateral total frontal lobe sparing one or both face areas of the precentral motor cortex (Travis and Woolsey, 1956) or the bilateral 4 and 6 preparation (Bieber and Fulton, 1938) produces reflex grasping. Of these operations, only the bilateral frontal lobe ablation presents inclusion of tonic innervation of the flexors of the elbow and of the knee with the grasp (compare Figs. 9f, 10c with 8j; Travis and Woolsey, 1956).



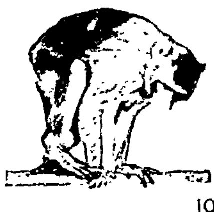


PLATE I

PLATE. I. Photographs of two macaques (*Macaca mulatta*), H 26 and T 55. H 26 was 12 years of age when the left Brodmann's area 4 (sparing the face area) was removed, December 16th, 1936. The right Brodmann's area 4 (sparing the face area) was removed May 11th 1938.

VII. PROGRESSION

Quadrupedal progression has been shown to be independent of the neocortex (Travis and Woolsey, 1956), though without the neocortex such progression is greatly modified. This is an achievement; for hitherto, bilateral frontal lobe removal seemed to prevent relearning to walk (Hines, 1943). The performance is, however, far, far from normal, more abnormal of course than that which characterizes the bilateral frontal lobe monkey (compare Fig. 10c with 16d; Travis and Woolsey, 1956). Even after bilateral ablation of the two motor cortices of the frontal lobe, the legs not the arms initiate diagonal quadrupedal progession. The quadrupedal progression in these monkeys is distinguished from that of the normal by a great limitation of retraction of the legs, and of protraction of the arms. The steps are short, the animal trips on

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T 55 ultimately became a bilateral 4 and 6 preparation (sparing the face areas). The operations were performed as follows: Left area 6 removed, June 16th, 1939; left area 4, October 19th, 1939; and right areas 4 and 6, October 24th, 1940. She was able to oppose the extended right thumb to an extended index finger, in spite of the removal of all cortical tissue even to the depth of the central fissure. On the left, she could with a flexed thumb touch a flexed index finger. The remaining three fingers were also flexed. On August 20th, 1942, I opened her skull and found that a minute strip of tissue which at that time lay anterior to the central fissure responded to electrical stimulation with extension of the thumb and extension of the first finger. More dorsally, flexion of the elbow and retraction of the arm were elicited. This strip of tissue was removed and with it vanished the ability to oppose the thumb to an extended index finger.

Fig. 7. Photograph of H 26 taken October 23rd, 1940. She is holding a small piece of fruit between an adducted left thumb and flexed index finger. The shoulders are raised because of the increased tone in the M. trapezius. Although she could flex the elbow, she flexes her head on a flexed trunk to meet a [illegible] arm, because prot[illegible]

Fi[illegible], the associated mov[illegible], which accompanies voluntary turning of the head to the right. The left arm is abducted and extended. Note that she is able to place her feet on the seat of the examining chair. Her toes are more extended than flexed.

Fig. 9. Photograph of T 55 taken on March 14th, 1941. showing that she is able to take a raisin from the hand of an examiner by flexing her thumb [illegible] flexed index finger. The other three [illegible] ment by retraction of the upper arm a[illegible] the arm to a greater degree than she could protract it.

Fig. 10. Photograph of T 55, taken May 12th, 1941, showing the difficulty experienced in turning to the right on a small base. The right foot is lifted but the left foot did not move until after the photograph was taken.

his own feet because the adduction of the legs (bifrontal) and/or the adduction of the arms (bifrontal plus one or both parietal lobes) do not allow one extremity to bypass the other.

VIII. TONE IN SKELETAL MUSCLE

Tone in skeletal muscle is assessed in several ways—by direct palpation, by increased resistance to passive movement, and by briskness of the myotatic reflexes. The brisk tendon reflexes may recruit the contraction of more proximal muscles and the stretch of some of these hypertonic muscles may be followed by clonus.

In the monkey, an excess of standing tone is present in the extremities contralateral to an ablation of the anterior part of area 4, of the whole of area 4, or after removal of the anterior lobe of the cerebellum. Cutting the medial reticular formation in the medulla oblongata or division of the ventral part of the lateral funiculus in the spinal cord or a ventral hemisection of the spinal cord (Hines, unpublished) increases tone in the contralateral extensors; whereas cutting the dorsal roots of S_1 and S_2 unilaterally in this beast causes excess of tone in the ipsilateral extensors.

Light ether anesthesia allows the appearance of an excess of tone in skeletal muscle, which sometimes involves flexors, sometimes extensors. Decrease in standing tone, i.e. inhibition of tonic extension, is obtained by stimulation of the anterior division of area 4, whereas inhibition of tonic flexion and release of the grasp is evoked from area 6 (Fig. 6). The inhibitory action against standing tone is predominantly contralateral and always more effective on the arm than on the leg, regardless of the site of the stimulating electrode. Anterior to this region in area 6 the inhibitory action against tonic flexion is effective bilaterally, contralaterally, or ipsilaterally and forms no predetermined sequence. Release of the grasp is frequently a separate event and its locus is restricted on the lateral surface to the anterior border of area 6 (Hines, 1943).

The results of differential ablation of these two areas are in a way reciprocals of each other. For removal of the area from which release of the grasp is obtained by electrical stimulation is followed by appearance of the grasp reflex stronger and more enduring in the contralateral hand than in the foot (Richter and Hines, 1934). Removal of the anterior border of area 4, stimulation of which decreased standing tone, is followed by exaggerated standing tone, brisk and irradiating tendon reflexes, hypertonus of the clasp-knife type (distributed in the

typical differential manner), clonus, and a minimal residual paralysis (Hines, 1936, 1937, 1943).

Standing tone is decreased after removal of the posterior part of area 4, of a part of or the whole of the parietal lobe, or of the hemisphere of the cerebellum. Surgical division of one pyramid (Tower, 1940) gives a generalized decrease in tone contralateral to the lesion as does severance of the lateral part of the basis pedunculus (the extensors of the digits (Cannon *et al.*, 1944) are not affected by the latter operation); cutting bilaterally the facilitators of tone in the lateral part of reticular formation causes a great decrease in tone of skeletal muscle (Ward, 1947). Hemisection of the medulla caudal to the pons (Niemer and Magoun, 1947) or division of the dorsal half of the lateral funiculus which includes the corticospinal tract was followed by decrease in tone of skeletal muscle opposite the lesion (Cannon *et al.*, 1943).

Surgical interventions which produce hypotonia show little differential distribution. The hypotonic states which follow pyramidal lesions, ablations of the cerebellar hemispheres (Botterell and Fulton, 1938), or removal of the parietal lobes effect muscles of the girdles and the proximal muscles of the extremities a little more than they do the distal muscles, except perhaps after removal of the parietal lobe, when the flexors of the wrists and fingers, and the extensors and the adductors of the fingers are especially hypotonic (Peele, 1948).

The differential distribution of increased tone so characteristic of the spastic state of spastic-hemiplegia in man is produced in the monkey by removal not only of the anterior division of Brodmann's area 4 (Hines, 1936) but also of the whole of area 4 (Hines, 1937). The hypertonia after the first lesion is maximal in the flexors of the elbow, the extensors of the knee, and the adductors of the thigh. The removal of total area 4 adds an appreciable hypertonus to the retractors and adductors of the upper arm, to the ventral flexors and the ulnar flexors of the wrist, and to the flexors of the fingers. In the leg, the increase in tone is found in the protractors and adductors of the thigh, in the ventroflexors and in the evertors of the ankle (Hines, 1937). The ablation of the whole of the supplementary and precentral motor cortices presents a comparable differential distribution of tone contralateral to the lesion, except that resistance to protraction of the leg rather than to retraction was found; and the toes resisted flexion (Travis and Woolsey 1956). Bilateral lesions increased the degree but did not change the distribution of tone in these muscles on the side of the last ablation.

Lesions involving one supplementary motor cortex only, produced increased tone in the above muscles of the arm and sometimes in three groups of muscles of the leg, the protractors and the adductors of the thigh and the ventral flexors of the foot (Travis, 1955b). Again, bilateral lesions give a greater increase in tone, bilaterally, than does a unilateral ablation upon contralateral musculature; but excess tone characterizes the flexors, not the extensors of the knee. Indeed, the clasp-knife type of resistance was described only once; and yet it is characteristic of the spastic state in man and in the monkey when the anterior part of or the whole of Brodmann's area 4 is removed.

Clonus did not succeed removal of the precentral motor cortex, or of the supplementary motor cortex (Travis and Woolsey, 1956), or of the parietal lobe (Peele, 1944), or of the cerebellar hemisphere. None is found after any type of lesion which produces hypotonia, although tremor does occur after removal of the cerebellar hemisphere when the nucleus dentatus is included (Carrea and Mettler, 1955). Clonus is found subsequent to ablation of the anterior half of area 4, or of the whole of area 4, or to that of the combined removal of the supplementary motor cortex and the precentral motor cortex. After the latter combined operation, clonus appeared in the flexors of the fingers and in the ventroflexors of the ankle; whereas after a unilateral removal of either one of the former two, clonus could be elicited by sudden and maintained stretch contralateral to the lesion in the quadriceps femoris, the gastrocnemius-soleus group, the adductors of the thigh, the tibialis anterior, the peroneus longus, and the flexors of the toes. In the arm, clonus was more difficult to demonstrate; it was often found in biceps brachii, in the long flexors of the wrist and fingers, and rarely in the triceps brachii.

Tendon reflexes were brisk opposite the removal of the anterior part of area 4, of the total area 4, and of areas 4 and 6. In the former two preparations, the greater the relaxation, the brisker were these reflexes. In the latter, the greater the relaxation in a quiet environment, the less brisk these reflexes became. After the former two types of removals of area 4, the tendon reflexes were found to be brisk in the flexors of the fingers, the M. flexor carpi ulnaris, the M. brachioradialis, the Mm. biceps and triceps brachii, the extensors of the knee, the flexors of the knee (i.e. the hamstrings), the ventroflexors of the ankle, and the M. tibialis anterior; and after the removal of anterior area 4 only, briskness in the flexors of the toes was added.

These brisk and hyperactive tendon reflexes frequently recruited others which after birth involved more proximal muscles. Contraction of M. quadriceps femoris was accompanied by a strong contraction of the adductors of the thigh on the same or opposite side. The flexors of the ankle irradiated to the flexors of the knee, while the tendon reflexes of the flexors of the digits might spread to some or all of the flexors of the extremity concerned. The M. biceps brachii has recruited the adductors of the upper arm.

In general, removals of the precentral arm area and of the precentral leg area plus slight injury to the supplementary leg area (on the lower bank of the sulcus cinguli) produced hyperactive tendon reflexes (triceps and flexors of the fingers) without any sign of hypertonia in the leg. Unilateral removal of the precentral arm area alone was followed by hyperactive tendon reflexes in the triceps and in the flexors of the fingers without palpable resistance to passive movement (Travis, 1955a). On the other hand, increased resistance to retraction of the leg and to extension of the knee was reported present subsequent to bilateral excision of both supplementary cortices in one monkey. Only one tendon reflex in the leg was listed as hyperactive, that of the flexors of the toes. Greater spread of resistance to passive movement characterized the upper extremity in this animal. Nevertheless, only the triceps and finger jerks were brisk (Travis, 1955b).

A different partition of the facets of spasticity marked the results of unilateral partial surgical division of the peduncle (Cannon *et al.*, 1943) and of two minute nicks in the white matter of the monkey's spinal cord (Wagley, 1945). Opposite the first lesion, tone was decreased and tendon reflexes were hyperactive without clonus. A cut into the ventral funiculus at T, secured on the side of lesion a minimal increment in tone, clonus or tremor, and brisk irradiating reflexes; one in the ventral part of the lateral funiculus at the same level produced a differential distribution of resistance to passive movement, brisk and irradiating tendon reflexes on the ipsilateral side, and no clonus. A similar partition of the elements of spasticity occurred during the postnatal development of the infant macaque. The differential resistance to passive movement disappeared in time before the tendon reflexes had ceased their irradiation, and recruitment disappeared before all the tendon reflexes had assumed a normal status.

The tendon reflexes are pendular, exaggerated only in the arc of their excursion, after pyramidal section (Tower, 1940), after removal

of the parietal lobe (Peele, 1944), as well as in the hypotonic state subsequent to ablation of the cerebellar hemispheres. In the pyramidal macaque, the tendon reflexes were slow, full, and unchecked. The ankle recruited the flexors of the knee. After removal of the postcentral gyrus and the posterior half of the precentral gyrus (Brodmann's area 4), the tendon reflexes were full, free, pendular, and recruited proximal muscles (Hines, unpublished).

In conclusion, the fact that stimulation of the lateral surface of area 6 or of the anterior border of area 4 evoked respectively inhibitory action against tone in the flexors or in the extensors, strongly suggests that localization of inhibitors of tone is not confined to the medial surface of areas 4 and 6 (Travis, 1955b). The addition of ablation of the precentral motor cortex to that of the supplementary motor cortex shifts the distribution of tone in the leg from the flexors to the extensors. Certainly, this finding insinuates that the former cortical area contains inhibitors of tone.

The evidence presented should persuade the most recalcitrant (but it will not) that facilitatory and inhibitory systems, separated from the corticospinal systems, can be activated in the reticular formation. Besides these fiber tracts clearly separated anatomically from the corticospinal tracts, there are others which are not. When the corticospinal systems are severed in the pyramids or spinal cord, either facilitatory systems are divided or facilitation is a part of the motor activity of these descending systems. Although the inhibitors of tone and movement are located in the midbrain tegmentum, a few inhibitory fibers lie dorsal to the pes pedunculi (Cannon *et al.*, 1944), continue caudalward into the pons with the corticospinal tracts (Tower, 1942), and become located in the reticular formation dorsal to the level of the pyramids.

The inhibitors of tone are a system apart from the corticospinal system, for the state of hypotonia caused by surgical division of the pyramids can be transformed to hypertonia by two types of additional operations, one upon the cerebral cortex, removing the anterior half of area 4 or the whole of area 4 (Hines and Tower, see Hines, 1943) and the other upon the spinal cord, cutting preferably the ventral half of the lateral funiculus (Wagley, 1945).

IX. PHASIC MOVEMENTS

The phasic activity of experimental animals can be assessed (1) by coordinated responses of the extremities to external stimuli—the

placing, stepping and hopping reactions of Rademaker and (2) by observation of spontaneous or self initiated movements. The coordinated movements of placing, stepping, and hopping are responses to tactile, proprioceptive, and visual stimuli.

X. COORDINATED MOVEMENTS AS RESPONSE TO SENSORY STIMULI

Placing is a quickly executed slight retraction followed by protraction which lifts the leg over the edge of the table. Slight extension at the knee is followed by slight retraction of the leg and flexion at the knee, with almost simultaneous ventral flexion at the ankle. Contact is made on the digital and interdigital pads. The toes are loosely extended and slightly abducted. Placing with the arm requires similar adjustments at the girdle, i.e. retraction and protraction of the upper arm. With the retraction, the elbow is flexed; with the protraction, it becomes extended. The wrist is dorsoflexed and contact is made by the digital and interdigital pads. The thenar and hypothenar eminences do not touch the surface, unless the monkey pauses to rest.

The position of the extremities when stepping or hopping has much in common with the final position assumed by the extremity when contact with the surface has been achieved. The normal adult macaque keeps up with the examiner's pace of transporting him through space, maintaining contact with the digital or interdigital pads of hand or foot. In the forward direction, the extremity alternately protracts and retracts in adduction. At maximum protraction or retraction, the second joint is about 160° extended. In between the maxima, the elbow or the knee is flexed and the ankle moves from a 90° to a 40° or less dorsoflexion. The retractors initiate stepping backward. The steps are shorter than in forward stepping and the extremity remains more flexed than extended. The contact of the foot or hand is made when dorsoflexion of these members is great. When the leg is in the protracted position, these distal joints are less dorsoflexed. Stepping to the right or left presents reciprocal images of each other. In stepping to the right, for example, the movement is initiated by the abductors of the leg, without changing the flexion at the second joint or at the distal one. The left leg follows quickly by protracting and adducting the thigh in one movement, so that as the advancing extremity crosses the right, it makes contact lateral and anterior to the locus momentarily occupied by the right foot. The arms follow a similar routine, except that the adducting extremity does not invariably cross over the abducting one.

The sequence of contraction of skeletal muscle which characterizes hopping is similar to that described for stepping except that the degree of adduction is rarely great enough to meet the midplane of the body.

These reactions must meet three criteria to be judged normal. The movements must be (1) quickly, easily, and smoothly performed (2) in correct sequence without disintegration and (3) contact with the surface must be light and without hesitation. The number of steps or hops per meter varies with the individual animal. He establishes his own norm, for postoperative comparison.

No deviation from the normal mode of placing was reported after lesion of the precentral gyrus or of the supplementary motor cortex (Travis, 1955a, b) nor after a small injury in the ventral funiculus of the spinal cord at T_7 (Wagley, 1945), nor after a complete ventral hemisection at that level (Hines, unpublished). Placing disappeared from the extremities contralateral to removal of Brodmann's area 4 (Hines, 1937), to removal of the frontal lobe (Travis and Woolsey, 1956), and to a cut into the dorsal half of the lateral funiculus (Cannon *et al.*, 1944). After unilateral pyramidal section, the whole movement is never executed by the contralateral extremities, and only fragmentary parts of the movement occur. Subsequent to a unilateral combined lesion of the precentral and supplementary motor cortices, placing was absent from the contralateral extremities until after comparable operations were performed on the other side. After 2 months, placing was "grossly defective" in all four extremities, but greatly facilitated by excitement. After removal of the parietal lobe, tactile placing disappears, proprioceptive placing returns, and visual placing remains. The motor response was "slower than normal in initiation and execution and frequently awkward" (Peele, 1944).

Given time for recovery unilateral lesions of the precentral motor cortex or of the supplementary motor cortex, hopping was reported to be normal. Stepping was not tested (Travis, 1955a, b). Small lesions in the ventral funiculus or in the ventral part of the lateral (Wagley, 1945) or a complete ventral hemisection at T_7 (Hines, unpublished) did not change the mode of the stepping or of the hopping reactions. But when a lesion in the lateral funiculus transgresses upon the site of the lateral corticospinal tracts, the act of stepping or of hopping is modified by inability of the retractors and adductors of the leg to contract.

Conjoint removals of the precentral and supplementary motor cor-

tices is initially followed by the inability of the contralateral extremities to hop at all; later, however, hopping forward and lateralward is accomplished in a crude fashion (Travis and Woolsey, 1956). Unilateral ablation of Brodmann's area 4 gives a similar result, for both stepping and hopping, medially and backward, by the extremities opposite the lesion has vanished. If these removals become bilateral, the arms refuse to step or hop (Hines, 1943).

Unilateral frontal lobectomy caused disappearance of contralateral hopping; bilateral, the hopping forward and lateralward reappeared and became eventually similar for each leg, and infrequently for each arm. The manner of use was not described (Travis and Woolsey, 1956).

The effort necessary to innervate contralateral musculature after surgical division of one pyramid made the initiation of hopping difficult. Like placing the hopping was fragmentary and ceased after a few efforts because of fatigue. Speeding the translation of the animal over the table improves the sequence of the movements of the legs forward and lateralward. The arms do not attempt to move.

The most severe loss of these coordinated adjustments occurred after cutting bilaterally the nerve fibers in the site of the corticospinal tracts (T_7). All hopping disappeared; only forward stepping remained. The toes turned under and the ankle was incompletely dorsoflexed.

These coordinated adjustments to somaesthetic and visual stimuli are not greatly modified when the extrapyramidal systems in the spinal cord are interrupted. Rather, they become markedly changed whenever the corticospinal systems are severed in the pyramids or in the spinal cord. The loss of large cortical areas does not obliterate either stepping or hopping completely in the lower extremity, although these losses may do just that for the upper extremity. The lower extremity does lose its ability to step or to hop backward or toward the medial plane of the body. The performance of the leg in stepping or hopping forward or lateralward can be said to be present, but the finer adjustments of dorsoflexion at the ankle, the loose easy extension and slight adduction of the toes are gone. Contact with the surface is no longer made by the digital and interdigital pads. The heel lands heavily and the toes no longer orient to the surface.

XI. SPONTANEOUS OR SELF-INITIATED MOVEMENTS

The mass organization of muscle (Tower, 1940, 1949) which survives bilateral surgical division of the pyramids in the monkey depends upon

the intactness of extrapyramidal systems. These extrapyramidal motor systems have not been specifically identified as yet. That the use of musculature made by the bilateral 4 and 6 (Brodmann's areas) preparation is similar to that made by the bilateral pyramidal monkey suggests that, aside from the difference in muscle tone in the animals, cutting the parietospinal fibers has not added any factor to the loss of discrete use of skeletal muscle. The temporal [illegible] in the cat have b[illegible] medullated [illegible] corticospin[illegible] fail to [illegible] be assayed by cutting them [illegible] with those from the frontal and parietal lobes.

Surgical division of the pyramids reduces the adult monkey to an *infantile age of 7 days, a time at which the* [illegible] *abled* the infant to get hold [illegible] This *act is prepared f*[illegible] followed by a limited pro[illegible] extension of the elbow, with the forearm in incomplete pronation. The hand is brought down upon the object with fingers partially flexed. Should the position of the desired object be shifted before this act is complete, the movement continues to the end; for a new orientation has to be made and another similar sequence of contraction of skeletal muscles has to be originated. The threshold for initiation of these movements is high; their activity easily fatigued (Tower, 1940, 1949).

In a similar manner, the 4 and 6 monkey (Hines, unpublished), in preparation for taking an object, retracts the upper arm, flexes the elbow, then protracts the upper arm, extends the elbow with the forearm *in almost complete pronation with the* fingers *equally extended*, and reaches the object (Fig. 9). The fingers flex about it and the arm returns to the original position of preparation. During the progress of these mass movements, the opposite hand grasps the cage bars or any other object for support. If the monkey di[illegible] necessary to reach [illegible] She makes h[illegible], leans slowly forward, changing her grasp to the side of the cage as she progresses, holds the mesh, and takes the object from the observer's hand with her mouth. Slowly she retraces her grasp upon the cage mesh, sits up, back curved, leaning against the cage or steadying herself by grasp upon the cage bars. She takes the object out of her mouth and holding it be-

tween equally flexed fingers and thumb, meets by flexion of the head the limited protraction of the arm.

The bilateral area 4 preparation (Brodmann) does not move into position to initiate this act. Rather, that animal is able to begin the movement by slight protraction of the upper arm, followed by extension at the elbow, incomplete pronation of the forearm, equal flexion of the fingers, and flexion of the thumb. After grasping the object, the upper arm is retracted, the elbow flexed the forearm in the 90° pronation / supination position. If the object is small, it will be held between a flexed index finger and an adducted thumb (Fig. 7). The flexion of the elbow will meet a head, flexed upon a flexed trunk.

Each of these three preparations starts the rhythmic movements of scratching at the girdles. No readjustment is made at the knee, the aim is poor. If the right spot is not met, readjustment must be started again at the girdle. Initiation of all movements is slow, and their execution fatiguing. Beginning in the proximal muscles, the sequence of contraction flows down the arm similar to "the march of movement" described by Hughings Jackson for man after severe injury to "the motor aspect of the mind" in the frontal lobe.

In these preparations, proximal initiation of movement remains, distal initiation is gone. The prime movers do not begin a movement, the synergists (for example, the wrist extensors) are not as active, and the antagonists not as greatly contracted as in the intact monkey. As a consequence, the flexion of the fingers over an object is not as strong; for when the fingers flex over an object, the wrist is flexed, not extended as it is in the normal monkey.

Fixation of the lower extremity and discrete use of the muscles of the lower extremity vanished when both lateral corticospinal systems were severed at T_7; whereas no loss of the use of skeletal muscle followed ventral hemisection at that level. Rather, all of the facets of spasticity appeared and the monkey although capable of normal use of skeletal muscle was reluctant to do so.

In the developmental maturation of the discrete use of skeletal muscle, the infant macaque moves the proximal muscle into position and then innervates the more distally placed muscles (Hines, 1942). During the time span when this was a common occurrence, it was possible to obtain fixation as a separate event by stimulating the lateral surface of area 6 with the 60 c.p.s. sine wave current. Similarly, during this same time in postpartum development, the electric current is able

to elicit contraction of a muscle plus fixation of more proximally lying muscles, preceding or accompanying the contraction (Hines and Boynton, 1940). The locus of this stimulus was topically typical of the muscles which contracted actively, not of those which were fixed.

In summary, after each of the above operations which destroy the corticospinal tracts, fixation of proximal musculature disappears and with it goes the discrete use of certain muscles. For example, subsequent to bilateral ablation of area 4 some of these muscles, which were used to initiate movement, can be used only in a sequence of movement; others cannot. Still others which are able to initiate movement present a limited degree of contraction.

This selective and peculiarly baffling paralysis falls naturally into three categories: (1) complete loss of use of muscle or paralysis, (2) loss of ability of muscles to act as prime movers although able to participate in generalized movement, and (3) retention of initiation of movement, although the degree of contraction is less than normal. Opposition of the thumb; flexion and extension of individual fingers; flexion and extension of toes; opposition of the hallux; dorsoflexion, ulnar flexion, and radial flexion of the wrist; dorsoflexion and eversion of the ankle, and adduction of the thigh and upper arm are gone. The flexion or extension of all fingers; adduction of the thumb; ventroflexion of the wrist; pronation and supination of the forearm; and ventroflexion and inversion of the foot at the ankle are a part of generalized, sequential movements. A few proximally placed muscles retain the ability to initiate movements, although the degree of their contraction is less than normal. These muscles are the extensors and flexors of the arm and leg, protractors of the leg, retractors of the arm, and abductors of the arm and of the leg.

Therefore, the selection of the prime movers, the action of the antagonists and synergists, as well as fixation at the girdle or trunk seem to be under the control of the descending systems which pass through the pyramids, because these activities disappear whenever the corticospinal systems are bilaterally interrupted. Further evidence for this generalization is given by electrical stimulation of one cortical surface after the ipsilateral pyramid has been cut. Under this condition, the electrode on the motor cortex does not evoke (1) contraction of single muscles or parts of muscles, (2) contractions of flexor or of extensor sheets of muscles, nor (3) sequential contractions which resemble the use patterns characteristic of the animal (Hines, 1940). Although fixa-

tion has been elicited by stimulating area 6 as a separate event and by stimulating area 4 as preceding or accompanying a movement, neither Dr. Boynton nor myself were able to obtain any type of fixation subsequent to surgical division of a pyramid. The phasic qualities of the discrete use of skeletal muscle are dependent upon the intactness of the corticospinal systems. Furthermore, without these important descending systems, initiation of movement is difficult and slow, aim is poorly realized and the monkey is unable to change his mind after a movement has been initiated. Indeed, the extrapyramidal systems which remain are not able to compensate for this loss, even when given years (10) to do so.

XII. CONCLUSIONS

Skeletal muscle is controlled at the spinal cord level by the activity of two types of different endings, the Golgi tendon terminal and the equatorial endings on the intrafusal fibers of the muscle spindle. The Golgi endings have a high threshold to stretch and are sensitive to the tension of contraction. The equatorial ending of the spindle is very sensitive to stretch. This ending can be activated directly by stretch of the muscle which contains it or indirectly by the activity of small nerve fibers which innervate the intrafusal fibers of the spindle. This arrangement is known as the gamma(γ) loop.

The cephalad centers of the central nervous system control skeletal muscle by direct innervation of ventral horn cells or by discharges to the intrafusal fibers which fire the equatorial sensory endings. The gamma loop of the spindle is accessible to electrical stimulation not only of facilitators and inhibitors of tone, found in the reticular formation, but also of the motor cortex, of the cerebellum, and of the nerve fibers within the pyramids (cat; Granit, 1956).

The normal regulation of small nerve activity to the spindle of skeletal muscle is the result of a combination of different discharges from the skin, muscle, and possibly the deep structures. This combination of exteroceptive and proprioceptive discharge may be a part of a peculiarly important pattern in the regulation of activity of skeletal muscle (Hunt, 1951).

The results of this analysis of the use of skeletal muscle by the macaque cannot be transferred directly to man. Rather, they are only suggestive. Although, in general, comparable results follow comparable lesions, there are several instances in which supposedly similar lesions

to elicit contraction of a muscle plus fixation of more proximally lying muscles, preceding or accompanying the contraction (Hines and Boynton, 1940). The locus of this stimulus was topically typical of the muscles which contracted actively, not of those which were fixed.

In summary, after each of the above operations which destroy the corticospinal tracts, fixation of proximal musculature disappears and with it goes the discrete use of certain muscles. For example, subsequent to bilateral ablation of area 4 some of these muscles, which were used to initiate movement, can be used only in a sequence of movement; others cannot. Still others which are able to initiate movement present a limited degree of contraction.

This selective and peculiarly baffling paralysis falls naturally into three categories: (1) complete loss of use of muscle or paralysis, (2) loss of ability of muscles to act as prime movers although able to participate in generalized movement, and (3) retention of initiation of movement, although the degree of contraction is less than normal. Opposition of the thumb; flexion and extension of individual fingers; flexion and extension of toes; opposition of the hallux; dorsoflexion, ulnar flexion, and radial flexion of the wrist; dorsoflexion and eversion of the ankle, and adduction of the thigh and upper arm are gone. The flexion or extension of all fingers; adduction of the thumb; ventroflexion of the wrist; pronation and supination of the forearm; and ventroflexion and inversion of the foot at the ankle are a part of generalized, sequential movements. A few proximally placed muscles retain the ability to initiate movements, although the degree of their contraction is less than normal. These muscles are the extensors and flexors of the arm and leg, protractors of the leg, retractors of the arm, and abductors of the arm and of the leg.

Therefore, the selection of the prime movers, the action of the antagonists and synergists, as well as fixation at the girdle or trunk seem to be under the control of the descending systems which pass through the pyramids, because these activities disappear whenever the corticospinal systems are bilaterally interrupted. Further evidence for this generalization is given by electrical stimulation of one cortical surface after the ipsilateral pyramid has been cut. Under this condition, the electrode on the motor cortex does not evoke (1) contraction of single muscles or parts of muscles, (2) contractions of flexor or of extensor sheets of muscles, nor (3) sequential contractions which resemble the use patterns characteristic of the animal (Hines, 1940). Although fixa-

tion has been elicited by stimulating area 6 as a separate event and by stimulating area 4 as preceding or accompanying a movement, neither Dr. Boynton nor myself were able to obtain any type of fixation subsequent to surgical division of a pyramid. The phasic qualities of the discrete use of skeletal muscle are dependent upon the intactness of the corticospinal systems. Furthermore, without these important descending systems, initiation of movement is difficult and slow, aim is poorly realized and the monkey is unable to change his mind after a movement has been initiated. Indeed, the extrapyramidal systems which remain are not able to compensate for this loss, even when given years (10) to do so.

XII. CONCLUSIONS

Skeletal muscle is controlled at the spinal cord level by the activity of two types of different endings, the Golgi tendon terminal and the equatorial endings on the intrafusal fibers of the muscle spindle. The Golgi endings have a high threshold to stretch and are sensitive to the tension of contraction. The equatorial ending of the spindle is very sensitive to stretch. This ending can be activated directly by stretch of the muscle which contains it or indirectly by the activity of small nerve fibers which innervate the intrafusal fibers of the spindle. This arrangement is known as the gamma(γ) loop.

The cephalad centers of the central nervous system control skeletal muscle by direct innervation of ventral horn cells or by discharges to the intrafusal fibers which fire the equatorial sensory endings. The gamma loop of the spindle is accessible to electrical stimulation not only of facilitators and inhibitors of tone, found in the reticular formation, but also of the motor cortex, of the cerebellum, and of the nerve fibers within the pyramids (cat; Granit, 1956).

The normal regulation of small nerve activity to the spindle of skeletal muscle is the result of a combination of different discharges from the skin, muscle, and possibly the deep structures. This combination of exteroceptive and proprioceptive discharge may be a part of a peculiarly important pattern in the regulation of activity of skeletal muscle (Hunt, 1951).

The results of this analysis of the use of skeletal muscle by the macaque cannot be transferred directly to man. Rather, they are only suggestive. Although, in general, comparable results follow comparable lesions, there are several instances in which supposedly similar lesions

do not apparently give similar results. Cutting the lateral two-thirds or four-fifths of the cerebral peduncle produced in the monkey a hypotonic paralysis with brisk tendon reflexes (Cannon *et al.*, 1944). Not so, in man. For surgical division of the lateral two-thirds to four-fifths of the cerebral peduncle for hemiballismus or Parkinsonian tremor stopped the pathological movement and left a few of the patients with "little paralysis" (how little, or what was paralyzed, was not mentioned) and with no increase in their previous spastic state (Bucy, 1957; Walker, 1952, 1955). At present there is no explanation of the variation of the degree of paralysis produced by apparently similar lesions. This inconsistency could be due to dissimilar preoperative brain damage, or to difference in the lesions which resulted from what seemed to be similar lesions, or to variations in the disposition of the corticospinal systems in the peduncle.

In man, as in the monkey (Travis, 1955b), no permanent paralysis follows removal of tissue in the medial surface (area 6) of the frontal lobe (Erickson and Woolsey, 1951); but, unlike the monkey, the change in tone is confined to the tonic innervation of the grasp, and again unlike the monkey, the intact supplementary motor cortex in man is apparently unable to prevent paralysis from following small ablations of the precentral gyrus. "If the hand area is completely removed, the hand becomes paralyzed for any skilled movement whatever" and "if the removal is small, the delicate movements of the fingers and thumb disappeared, although the movements of the digits altogether in flexion or extension and movements of the wrist, elbow and shoulder may be produced" (Penfield and Erickson, 1941). Foerster (1936a) also found that the muscles of the hand or fingers were not implicated where a paralysis of the shoulder, upper arm, and forearm existed. Conversely, a circumscribed loss of use of the muscles of the hand and fingers followed removal of the hand-finger area alone. After years of systematic training, the patient learned to hold a pen and write. Each movement of the right hand, however, was accompanied by a similar one of the left (normal). Foerster considered this relearning to be dependent upon the ipsilateral corticospinal tract. It is possible that the ability to supinate (45°) the forearm remained and if so it could be utilized in the retraining process.

Even a year after the ablation of the whole precentral gyrus, when Marinesco's (1903) patient picked up an object, the arm moved into retraction and flexed as preparation for protraction and extension. In

this movement, the wrist was in complete pronation and the fingers were extended. After touching the object, the fingers flexed tightly upon the object without the synergistic action of the dorsoflexors of the wrist.

Since isolation of the precentral gyrus, made by removal of all surrounding cortical areas (Penfield, 1954), is not followed by any deviation from the normal use of skeletal muscle, the "motor" cortex in man seems to be independent of interregional cortical connection. That the cerebello-thalamo-cortical system can give the information about the body necessary to "run" the precentral gyrus is doubtful. The new spinocortical system carrying proprioceptive sensibility to the motor cortex may have to be evoked, if of course, it is eventually discovered as an ascending system in the pyramids of man.

Strangely enough, large removals of cortical tissue are not counterindicated, for Welch and Penfield (1950) report that ablations of diseased tissue surrounding and even including the precentral gyrus (man) decreased the preoperative spasticity without increasing the paralysis. Similarly, subsequent to removal of a whole hemisphere for infantile hemiplegia, the resulting decrease in spasticity seems to free the extremities for greater use in some patients (Krynauw, 1950; Bates and McKissoch, 1951). This greater use rarely included a real improvement in that of the muscles of the hand, although many of these patients managed to walk with greater ease after the operation. Similarly, large ablations of normal cortical tissue (frontal lobe plus parietal lobe) from the macaque caused the upper extremity opposite the lesion to be demoted from an organ of exploration of space to that of a moving support, incapable of initiation of progression (Travis and Woolsey, 1956).

The final and minute analysis of the ability of the macaque to direct his skeletal muscle toward the achievement of a desired end (object visible or otherwise) awaits completion. We do know, however, that increased or decreased tone and facilitation of movement are contributions of descending systems not located in the medullary pyramids. Selection of prime movers, the activity of antagonists and of synergists are impossible in the absence of the corticospinal systems. Fixation, proximal to contracting muscles, that prerequisite of all discrete movement, is dependent upon the existence of the corticospinal systems which stem from the frontal lobe; fixation at the girdles, however, is dependent upon this corticospinal system and another from the parietal lobe as well as upon the lateral hemispheres of the cerebellum. The

mass organization of skeletal muscle innervated proximally survives injury to the corticospinal systems; whereas the discrete organization of that tissue, innervated distally, does not. Ease of initiation of all movements, the ability to reach for and take the desired object (without movement into position), the ability to stop sequential movements and to start that sequence again toward the same or another end, to split and utilize the synergies of mass movements—these are characteristic of the corticospinal systems' contribution to muscle use.

The facilitation of movement so necessary for ease of initiation, the shifts in degrees of tone from one muscle group to another as the intended sequence of movement develops, are dependent upon the extrapyramidal systems. These systems control the proximal initiation of contraction of muscles of the whole extremity, as flexor or extensor synergies. This use of muscle contributes force and smoothness to the resultant movement.

In the absence of the extrapyramidal systems, the remaining corticospinal systems are not able to raise the central excitatory state to the level where movement is easy to initiate. After loss of the corticospinal systems, the initiation of all movement is delayed, its progress slowly executed and its aim rarely realized. Fatigue is, indeed, great. Consequently, an accompanying emotional state transforms this picture of results of loss of the corticospinal tracts by raising the central excitatory state so that the latent period for beginning of the movement is shortened, the total sequence of the movement more quickly executed and the aim frequently realized. Fatigue, although present, is less. The hypokinesis of extrapyramidal lesion seems to be restricted only to the reluctance to start a movement. Once begun, the movement appears to be normal in ease of execution, in sequence of muscle contraction, and in manner of use of distal musculature. Fatigue, if present, is not apparent. Certainly, the corticospinal systems are able to utilize the lower centers of the brain stem and spinal cord to produce with the aid of the extrapyramidal systems those movements which distinguish man from other primates, and other primates from other mammals.

I have attempted to analyze the relationship of the central nervous system in the primate, particularly of the cortex cerebri via the corticifugal systems, to the control of the use of skeletal muscle. Muscle is that tissue which expresses our slowly and sometimes painfully achieved education and confers upon us, as men, the gift of communication.

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CHAPTER XII

Selected Topics on the Physiology of the Heart

W. F. H. M. Mommaerts, B. C. Abbott, and W. J. Whalen

I. INTRODUCTION

There seems to be no modern comprehensive treatise on the entire field of cardiac physiology. Yet, however much such a monograph would be desirable, it can hardly be the purpose of the present authors to supply it at this occasion, if for no other reason than that it might well occupy the full size of this volume. What will be attempted, instead, is a discussion of several selected currents in cardiac physiology. Some of these are chosen so as to provide some measure of continuity with the physiology of muscle as discussed in other chapters of this book, but some also will bring out major differences.

The vertebrate heart, and the mammalian heart in particular, is a closed organ which displays a much greater degree of physiological autonomy than individual skeletal muscles. While the latter are passive, and are brought into action only upon instruction from the central nervous system, the heart is an organ with inherent automatic activity. This, in the higher forms, is essentially a property of the myocardium itself, although in practice rhythmicity is seated in specialized muscle fibers located in the nodal tissues connected with a distributing system of Purkinje fibers. While skeletal muscle is dependent upon the voluntary nervous system, it does contain within itself certain elements for the regulation of its activity. The intrafusal muscle cells and the nerve fibers associated with them form a servosystem for the control of posture and for the smooth performance of voluntary contractions. Almost all physiological contractions are tetanic, and extensive grading of the strength or power of a muscle is possible by the recruitment of fewer or more motor units. Involuntary regulation, as exemplified by the reactivation of fatigued muscle by sympathetic stimuli (Orbeli effect) may

occur, but the extent to which this has physiological significance seems a matter of dispute. The heart, on the other hand, reacts in an all-or-none manner, always being recruited in its entirety, and it has, as far as known, no built-in servosystems for its regulation. One inherent regulatory mechanism consists of the manner in which its systolic strength depends upon the degree of diastolic filling, that is, the initial length of the myocardial fibers (Starling's law); then, the heart displays to a striking extent a dependence of its contractile force upon the previous cycle of activity, in the sense of the staircase phenomenon. Finally, the heart *in vivo* is under the involuntary control of its ortho- and parasympathetic innervation, and the neurohumors elaborated by these systems exert manifold effects. And, while single twitches occur only exceptionally in voluntary muscle *in vivo*, the heart does always contract in this manner, and cannot normally be made to summate its single contractions because the refractory period is of the same order of duration as its mechanical activity cycle.

With regard to metabolism associated with activity, it is often held that cardiac activity is strictly aerobic while skeletal muscle is not immediately dependent on oxygen provision. This distinction has practical validity when typical white muscles are considered, but it is likely that many red muscles which maintain steady activity (such as the pectoral muscles of predominantly airborne birds or bats) are closely comparable to hearts in this respect. Intimately related to this problem is the indefatigability of the heart, which functions permanently over a lifetime. The essential point is here that in the heart, as well as in other muscles which must work uninterruptedly for considerable lengths of time, the enzyme systems for respiration and oxidative phosphorylation are sufficiently developed to cover the metabolic needs under all, or nearly all, circumstances. To engage in an oxygen debt cannot solve any of the physiological needs in such a case, except for those limited periods of transition in which the activity is raised from a lower to a higher power, before the corresponding circulatory adjustment has been accomplished. But even for those brief periods, an oxygen debt seems to be avoided with the aid of oxygen stored by myoglobin.

Scattered discussion in the current literature deal with the question of whether the fundamental processes in the heart are "the same" as in muscle. We shall not belabor this point, mostly because the "sameness" is subject to various interpretations, and those processes might tend to be defined as "fundamental" which are demonstrably identical

in the two cases. Accepting a basic resemblance or identity of the contractile mechanisms, it is still clear that the architecture of the heart is so characteristic that many features of its mechanism and function will appear in a very special way. The special play of forces in a hollow organ; the alternation of isometric and isotonic contraction in the course of an activity cycle; the virtual absence of negative work before diastole; the different mechanical strains upon the auricles and ventricles and upon the left and right halves of the heart; the unique organization of the intracardiac circulation; the encapsulation within a relatively inextensible pericardium; and, in short, the anatomical complexity of the organ, all impose upon cardiac physiology a unique character. The study of the mammalian heart has, furthermore, been affected by the circumstance of having been studied in close contact with medical problems, whereas it also poses methodological requirements which have often discouraged its use for many of the epoch-making biochemical and biophysical investigations which have made the study of skeletal muscle such a prominent chapter of pure science.

Among the broad fields we have been obliged to omit altogether, we must mention the comparative physiology of this organ (Clark, 1927), (Prosser, 1950); the physiology of the coronary circulation (Gregg, 1950); the study of cardiac metabolism (Mommaerts, 1958); the description of the pressure pulses and their relation to hemodynamics (Wiggers, 1923, 1952); its excitability and its nervous regulations (Brooks *et al.*, 1955); and its electrophysiology in relation to the electrocardiograms (Hecht, 1957; Sodi-Pallares, 1956). Little, indeed, remains: mostly contractility as such and some remarks about automaticity. This choice is not entirely arbitrary, but is made because it might be called the "muscle physiology" of the heart. It is hoped that this introduction, and its references to the literature, may suffice to point toward the many fascinating problems which had to be omitted.

II. CARDIAC AUTOMATICITY AND ITS RELATION TO CONTRACTION

Among the numerous rhythmic activities in nature (Arvanitaki, 1938), the regular activity of the heart is one of the most striking and fascinating examples. Viewed from the standpoint of comparative physiology (Clark, 1927), the heartbeat is not, however, a process that has a uniform cause in all forms of animal life. Two distinct categories can be discerned: those forms in which cardiac activity is caused by the rhythmic discharges of associated neurons, and those in which nerve

elements are absent or in which, if present, their role is limited to a regulation or modification of an otherwise determined automaticity, which must therefore reside within the heart muscle itself. The classical example of neurogenic automaticity is the heart of Limulus which, according to Carlson (1904), becomes inactive when the adjacent ganglion is removed. The demonstration of the existence of myogenic rhythmicity results from studies on the heart of the chick embryo (Patten, 1949), which displays regular contractions before being invaded by, or associated with, nerve cells. However, recent improvements in histological techniques have thrown some doubt upon the justification of the assumed absence of nerves in embryonic tissues and other sites in the body, so that this argument may well need re-examination (Meyling, 1953; Nelemans and Dogterom, 1956).

The problem of neurogenic cardiac activity is currently being investigated by Bullock and his associates (Maynard, 1955; Bullock and Terzuolo, 1957) on the cardiac ganglia of lobsters. According to their beautiful analysis, the origin of activity resides in a cluster of nine ganglion cells which generates a complex pattern of rhythmic discharges. This ganglion cluster is of importance not only to explain the cardiac activity of the lobster, but is also, as a "micro-brain," of considerable general significance. In the vertebrate hearts, to which this discussion shall be limited, the activity does at least in part reside within the myocardial cells themselves. In the heart of e.g. the frog, automaticity is still closely linked with the intracardiac ganglia, which can be stepwise eliminated by the Stannius ligatures. In the case of warm-blooded animals, of which the mammalian examples are extensively investigated, the phenomenon is more exclusively a function of myocardial elements, some of which are specialized toward an automatic impulse generating and conducting system, which are described in the manuals of anatomy and histology. In its typical form, this system consists of a primary pacemaker, the sinoauricular (S–A) node; a secondary pacemaker, the auriculoventricular (A–V) node; and the Purkinje system or the bundle of His-Tawara with its two major branches and their further ramifications. Although this system is of myocardial origin, it is nevertheless instructive to compare it, functionally, to a small nervous system, in which the Purkinje fibers would correspond to the motor axons. Likewise, the inhibition of the automatic center by vagal impulses stands in formal analogy to the suppression of the activity of sensory receptors by special inhibitory effector

neurons (Burgen, 1955). Such analogies should not be belabored, yet it is of interest that, while the myocardium as such seems to contain an unspecific cholinesterase, the bundle of His contains the specific acetylcholinesterase in amounts comparable to that in peripheral nerves (Mommaerts *et al.*, 1953).

Instead of attempting to discuss the problem of cardiac rhythmicity within the restricted space available, it will be preferred to limit this discussion to two especially relevant points: the fundamental nature of automaticity and its relation to some properties of muscle; and the connection between bioelectrical and mechanical events. The broader problem of the electrophysiology of the myocardial cells and of the irritability of the heart is, fortunately, easily accessible through the excellent compilations by Brooks *et al.* (1955), and by Cranefield and Hoffman (1958), and, less recently, through the extensive work by Schaefer (1942), while the important work by Weidmann is available in monograph form (1956). The reader is referred to these sources for a general treatment of the field. Concerning a problem of practical importance, the relation of automatic activity to the electrocardiogram, reference is, in addition, made to special works like that by Sodi-Pallares (1956) or Bayley (1958). Special literature references in this section will only be made to papers of crucial importance to the text.

Like other irritable cells, those of the myocardium and its special derived tissues display a resting transmembrane potential of the order of 90 mv., the outside being positive with regard to the inside. Values for this potential in individual instances, as tabulated in the quoted summaries, usually vary relatively little, although certain reported figures are distinctly less. No general interpretation can be given for these deviating values, except perhaps for the case of the chick embryo (10–70 mv.) where it might conceivably be indicative of a gradual establishment of the characteristic differentiation of the intracellular electrolyte composition in the course of embryonic development like, conversely, the accumulation of potassium ions disappears during the loss of vital activities in the course of the specialization of erythrocytes in many species (Ponder, 1948). The action potential, as in other tissues, consists of a reversal of this potential with an overshoot of, generally 10 to 30 mv. (even in the case of the chick embryo heart, notwithstanding its lower value of the resting potential).

The actual shape of the action potential differs considerably from that in most other tissues, in that, after the spike potential itself, a

remaining depolarization persists over a considerable time as a plateau. While it appears to some extent arbitrary to distinguish between direct repolarization, a negative afterpotential, and a plateau, the fact remains that the plateau in cardiac cells is of striking magnitude and duration. Its course and extent differ for different cells in heart, but that for the Purkinje cells, according to Weidmann's (1957) studies (Fig. 1), can be used to illustrate some of the general features, except that

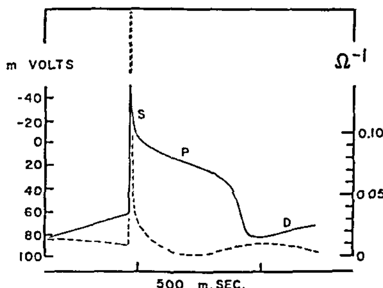


FIG. 1. The action potential of cardiac muscle (Purkinje fiber of kid heart). S, spike potential; P, plateau; and D, spontaneous depolarization phase. Broken line, changes in membrane conductance in arbitrary units, its value at the height of the spike being taken as unity. (Redrawn from Weidmann, 1957).

the slowly progressing depolarization preceding each individual spike is characteristic for those parts of the heart that display automatic activity. The same figure also shows the relation between depolarization and membrane impedance. Apart from the gross parallelism apparent during the spike potential, these two phenomena are not as closely parallel as would superficially be expected; a discussion of the connection between action current and impedance (Cole and Curtis, 1939) is outside the scope of this chapter, however.

The mechanism of rhythmicity becomes understandable in the light of a comparison with the way in which a skeletal muscle fiber is normally excited via its motor nerve fiber. According to Kuffler (1942) and del Castillo and Katz (1956), the arrival of a motor impulse at the neuromuscular junction gives rise to an end-plate potential which, es-

pecially if the receptor structure is partially inhibited by curare, can be seen to rise gradually until a critical level is reached, at which moment it changes explosively into the propagated action potential of the muscle fiber. It is typical for the automatic cells in the heart, according to Weidmann (Fig. 1), that a slow depolarization occurs gradually and spontaneously in the interphase between individual impulses. This process occurs at a characteristic velocity (e.g. 22 to 67 mv. per second), until the membrane potential is reduced to a threshold value of the order of 70 mv., at which point, by a process of self-stimulation, a conducted action potential results. Regions of lower rhythmicity differ from the pacemaker in that their spontaneous diastolic depolarization is slower, so that it is normally overtaken by an impulse arriving from the faster center. The metabolic and intricate nature of the slow diastolic depolarization is unknown, but it would seem that a slowly degenerating potential is no more mysterious than a constant one. The alteration of cardiac rhythmicity by autonomic stimulation seems to act directly upon these inherent mechanisms (Hutter and Trautwein, 1955). Vagal activity causes a slowing of the diastolic depolarization (or even a reversal in case of strong stimulation), while sympathetic stimulation accelerates the same depolarization process. Acetylcholine and epinephrine act in the same sense as in the corresponding nerve activities. The literature on these questions is reviewed by Cranefield and Hoffman. Some data begin to become available on the effect of several ions upon these same processes; their further study may be one of the ways to penetrate into the mechanism of the automaticity, as may the study of the effect of lack of oxygen, which enhances automaticity (Trautwein *et al.*, 1954). Not only the diastolic depolarization, but also aspects of the action potential are, of course, affected by several experimental parameters (e.g. Brady and Woodbury, 1957, and the quoted general literature).

The other problem to which we shall give brief attention is that of the relation between electrical and mechanical activity. In a broader sense, some facets of the relation between stimulation and mechanical response will be discussed in the last section of this chapter, devoted to the active state. Here, the said relation will be examined more empirically. Many investigations in this direction are affected by the circumstance that in work with locally stimulating electrodes and a microelectrode for recording the action potential, the latter registers the electrical response sharply, while the mechanical activity curve is

broadened by the effect of the finite conduction velocity. Only one investigation (Hoffman *et al.*, 1956) has employed multiple-point stimulation; in our current work laterally placed massive electrodes have been applied, but their actual effect remains to be investigated (compare Rushton, 1930). Due to these methodological uncertainties, time relations such as the latency period and the latency between rise time of the action potential and the onset of tension, are not yet definitively known. Investigations on the effect of certain factors upon the action potential and upon contractile force only show that the two are not rigidly related. Acetylcholine, e.g., decreases both the duration of the action potential and the contractile force; epinephrine increases both (Webb and Hollander, 1956) in certain preparations. Digitalis glycosides, however, shorten the action potential while strengthening the contraction (Woodbury and Hecht, 1952); several ions likewise have varied effects. Still, there exist circumstances under which, within a certain range of varying an experimental parameter, the length of the action potential may be determining the contractile force (Niedergerke, 1956). In view of the consideration given in a later section that increased contractile strength can be caused in three different ways (increased intensity, increased duration of the active state, and increased velocity of shortening of the contractile component) it is small wonder that no universal correspondence between force and action potential exists. Furthermore, while among these three parameters, at least the duration of the active state (if not the others) may be regulated by membrane events, it is not certain that the duration of the plateau is the most relevant index of the magnitude or extent of the membrane change. One might consider that the "plateau" of the action potential switches the active state on and off, but not enough data are available to discuss such a hypothesis fruitfully at this moment as a general explanation. In fact, the notion is contradicted by the finding that, while the plateau and the refractory period approximately coincide, the active state (Abbott and Mommaerts, 1958) has decayed before that time. Furthermore, such an assumption would grossly contradict our knowledge of skeletal muscle. There is frequently an approximate coincidence between the end of the action potential and the beginning of relaxation (Trautwein and Dudel, 1954), but under experimental conditions such as digitalis poisoning, or application of acetylcholine to the cat auricle (Burgen and Terroux, 1953), this coupling can be entirely removed. Only actual measurement of the active state can

provide a decision as to this connection, as will be discussed in Section V.

Concerning the biochemical aspects of automaticity and impulse conduction, mention has already been made of the finding (Mommaerts *et al.*, 1953) that the bundle of His contains acetylcholinesterase, about as much as e.g. the sciatic nerve. Similarly, Briscoe (1954) showed that the specific acetylcholinesterase predominates in the right rabbit auricle, while the left auricle, which has less automatic activity, is richer in nonspecific cholinesterase. It would be desirable to show with histochemical techniques that this enzyme is indeed located within the Purkinje fibers (or pacemaker tissue), and to extend, with investigations into other components of the acetylcholine cycle and with inhibition studies, knowledge of the relation of this enzyme to impulse conduction. Not unlikely, the conclusion will emerge that the conductive system in the heart is "cholinergic." What this means, even in nerve (Nachmansohn, 1955), is not entirely clear in point of detail, although it is important to emphasize that according to a study by Wilson and Cohen (1953) with enzyme inhibitors which can penetrate the living cell, the participation of acetylcholinesterase in the conduction process is indeed essential. Cholinergic mechanisms of a sort may, also, prevail in other parts of the heart even though here a nonspecific cholinesterase prevails. Acetylcholinesterase inhibitors do, indeed, suppress various manifestations of automaticity and impulse conduction in the heart (Burn, 1956). Mention should be made here of the important observations of Bülbring and Burn (1949) and Burn (1956), according to which the choline acetylating activity in acetone powders of isolated rabbit auricles was greatly reduced when these auricles had become spontaneously arrested, but was restored toward normalcy when the auricles' spontaneous activity had been restarted by acetylcholine. These authors, therefore, assume a relation between automatic activity and acetylcholine synthesis activity. Historically, this is reminiscent of Deuticke's (1930) observations on post mortem changes in skeletal muscle, in which such properties as the solubility of myosin were related to the "synthetic power" for sugar phosphates, later shown (cf. Mommaerts, 1950) to depend on the presence or absence of ATP. A similar explanation, the level of some essential coenzyme, may apply here too. At any rate, besides the well-known inhibitory action of acetylcholine upon the heart which can be suppressed with atropine, it may well also be intrinsically related to endogenous activation processes, presumably located in pacemaker tissues; in addition, there may

be enhancing effects upon contractility, acting by a nicotinelike effect upon adrenergic nerve elements (see Burn, 1956, and Section IV of this chapter). We recall, furthermore, that the heart contains a labile acetylcholine precursor (Abdon and Hammarskjöld, 1944). The complete separation and elucidation of these various roles and effects of acetylcholine will require considerable further work.

III. CARDIAC CONTRACTILITY

Although many processes and regulations contribute to the physiological architecture of the heart, its principal manifestation and sole function is its contractility. With regard to this activity, the atria and the ventricles are quite separate structures; we shall give preferred consideration to the latter in view of their more pronounced and essential mechanical performance. In the atria, due to the origin of automaticity in the sinoauricular node and the fairly homogeneous spread of the excitation wave from that point (at a speed of the order of 1 m. per second in the human heart), the contraction wave likewise spreads over the bulk of the auricles, following the excitation wave after a latency period of about 20 msec. Since the times of conduction, latency, and contraction are of comparable orders of magnitude, the resulting asynchronism of the activation of the different parts of the atria gives rise to a so-called fractionate contraction, in which the activity cycles of the various parts overlap, but not to the same extent as in the asynchronous tonus or tetanus of a skeletal muscle. A part of the spreading impulse-field encounters the auriculoventricular node. The excitation of the nodal tissue and the relatively slow propagation within it (0.2 m. per second) cause a delay of about 50 msec., after which the impulse is conducted with considerable velocity (3 to 5 m. per second) through the bundle of His and Tawara, reaching most parts of the ventricular muscle within 10 to 15 msec., after which it is conducted through myocardial cells at a lower rate (0.4 m. per second) over the remaining distances. Thus, ventricular systole is likewise of a fractionate nature, although not as strikingly asynchronous as in the atria. One aspect of the asynchronism is probably that the papillary muscles contract slightly in advance of the bulk of the heart, securing the tightening of the tricuspid and mitral valves during ejection. However, left and right ventricles contract at about the same moment. These time relations have been described in great detail by Lewis (1920) and Wiggers (1923, 1952) and, related to modern electrophysiological work, by Brooks *et al.* (1955).

While the systole of the myocardium is obviously analogous to the contraction of skeletal muscle, special problems arise from the complicated morphology of the heart, and from the fact that in a hollow organ, in accordance with Laplace's law, the tension exerted on or by the muscle elements is not exclusively determined by the pressure in the interior but also by the size of the enclosed cavity and the thickness of the wall. This will be discussed further below. It is also likely that in cardiac contraction, pressure is exerted not only by the musculature upon the fluid content, but also by different muscular layers upon each other, so that intramuscular pressure changes will occur during the contraction cycle and will periodically affect the intracardiac circulation. Internal pressure changes are not absent in skeletal muscle (Hill, 1948), but seem to depend exclusively on special arrangements of the fibers causing a compression of the interior, while for the heart the phenomenon would be an inherent one.

The pressure changes in the cardiac chambers throughout the cardiac cycle and their relation to the periodic propulsion of the blood have been elucidated by Wiggers and are described in all texts of human physiology (cf. Wiggers, 1928, 1949). The overall systolic process has the character of an afterloaded contraction in which two phases can be distinguished. In the beginning of the first phase, when tension begins to rise (the ascent of pressure is slow—Wiggers's entrant phase—due to the fractionate character of the contraction), the mitral and tricuspid valves close, while the semilunar valves are not yet opened. During the course of this phase, then, contraction is purely isometric, apart from the small deformation permitted by a change in shape of the ventricles around the enclosed volume of blood (cf. Rushmer, 1955a, b, concerning the change in shape during ejection). Isometric contraction continues until the pressure reaches the arterial pressure level, after which the semilunar valves open and the systole now enters the ejection phase which, but for the concomitant alterations in arterial pressure, is isotonic. After emptying, the semilunar valves close and diastole occurs at the relatively low pressure of the inflowing blood. Thus, the contraction cycle is a definite sequence of isometric and isotonic contractions and of a relatively unloaded lengthening, with notable absence of the phase of negative work so manifest in skeletal muscles, which start contracting actively while still in the process of forced lengthening by the action of antagonists or by the inertia of moving parts. Henderson (1906) has made an illuminating comparison between the heart and a

muscle operating a work adder in which a heavy weight, afterloaded, is lifted and supported by a ratchet wheel, whereupon the muscle relaxes while being stretched by a much smaller load.

A basic part of the description of the contraction of the heart is the relation between diastolic volume and contractile strength, just like the length-tension diagram of resting and active muscle summarizes major aspects of its mechanical behavior. The first description of cardiac contraction in terms of this analogy was given by Frank (1895), following the basic investigations on skeletal muscle by Fick and by Blix; Starling has also extensively used this comparison (e.g. Patterson *et al.*, 1914). More recent investigations are those of Reichel (1939a) and Lundin (1944). We shall consider a part of this problem in relation to the question how the intensity of cardiac activity is regulated. This is a highly complex situation, many angles of which, involving reflex mechanisms and other controls, cannot be treated here (see e.g. Stead and Warren, 1947; McDowell, 1938, 1956). But a part of the problem can be formulated in the question: how can the heart accommodate changing volumes of blood offered by the venous system, and how can it maintain its output against changes in peripheral resistance? This seems clearly answered by the classic work of Starling (1918; Patterson *et al.*, 1914), in which he enunciated the "law of the heart." In essence: when greater diastolic filling occurs, contraction will start from a greater initial fiber length and will, as predictable from the length-tension type of relations, develop more tension and perform more work per cycle. Thus, after a few transitory beats in which the required degree of filling is reached, the heart will again expel as much blood as enters into it. A similar response occurs when blood has to be propelled against an increased arterial pressure. During a few initial beats, incomplete ejection takes place, so that the retained blood adds itself to the new influx to give the greater diastolic filling which causes more forceful contraction. This adaptation works up to a certain optimal distension, after which work declines with increased filling. This important principle may be invoked in the physiological adaptation to pathologically diminished contractile power. The heart operates then at an increased diastolic volume and can do so successfully within reasonable limits. However, its reserve margin is diminished, and upon increased demand the over-optimal region (cardiac decompensation) may be reached. If the relation between work and diastolic volume were schematically described by a curve of the type N in Fig. 2, it may

be stated that the failing of hypodynamic heart operates on a lower curve F, where the contractile activity for a given fiber length is decreased. These relations can also be changed within non-pathological limits, and it appears that e.g., epinephrine can bring the activity to a more effective level as in curve E. However, there is uncertainty as to the limits to which these principles can be applied to the human heart *in situ*, partly because of the restrictive influence of the pericardium, and also for other reasons (e.g. Rushmer, 1955a, b; Sarnoff, 1955).

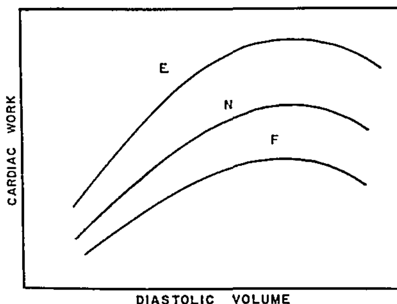


Fig. 2. Schematic diagrams of the relation between cardiac work and diastolic filling. If curve N represents a normal heart, F would be one in failure, and E would be one strenghthened by epinephrine or norepinephrine.

These problems, however, would fall beyond the limits of this chapter.

By and large, with increased work, cardiac metabolism increases *pari passu*. Consequently, Starling's law has been extended to state that the oxygen consumption of the heart is determined by its fiber length (or tension, for that matter). This problem has become considerably complicated, and reference must be made to a recent symposium on this relation (Katz, 1955; Sarnoff, 1955). Since even the question as to the effect of stretch upon the resting metabolism of skeletal muscle does not seem to be universally answered, we deem it wise to make no predictions about the future development of the highly complicated problem of the active heart.

Inasmuch as some of the preceding considerations are partly based

upon analogies between cardiac and skeletal muscle, it would be instructive to have mechanical studies on bundles or strips of myocardium, for direct comparison. Such measurements have been performed e.g., on ventricular muscle strips by Wöhlisch and Clamman (1936) and by Feigen *et al.* (1952), and on parallel-threaded muscle columns of the frog heart by Lundin (1944). We have currently studied some mechanical properties of papillary and trabecular muscle from the cat's heart, and refer to Fig. 3 for certain illustrative results. Length-tension

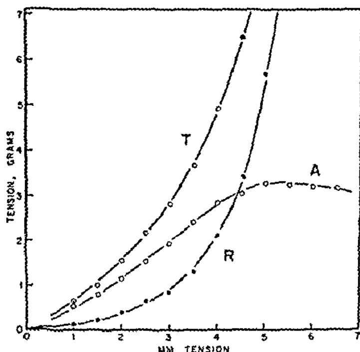


FIG. 3. Length-tension diagrams of cardiac muscle, according to original measurements on a trabecula carneae preparation of the cat. Muscle at 27°C. in oxygenated Ringer; length, 17 mm. at the point of origin; average cross section, 1.85 $mm.^2$; rate of stimulation, 1 per 5 sec. Separate curves for: R, resting tension; A, actively developed tension; and T, total tension.

diagrams for different preparations, within the limits of their variability, do in general seem to display a rather sharp difference in comparison with the same diagrams for typical skeletal muscles. The latter develop their maximal tension at a length equal to, or not in great excess of, *the resting or natural length at which passive tension is slight.* In heart muscle, there is a pronounced overlap of the regions of resting and active tension, and there is no clear optimum of the latter until, at very great initial extension, the active tension declines. Undoubtedly,

this distinctive property is of physiological significance, but for the discussions of this and other features it has to be kept in mind that in the heart, as a hollow organ, the relation between workload and tension is altogether different from that in a "linear" muscle. The literature on this significant subject is very limited (Woods, 1892; Burch, 1955a, b; Burch *et al.*, 1952; Burton, 1957), and the implications have not been fully worked out, but may be briefly indicated as follows:

The basic law of importance to our problem is that of Laplace on the relation between pressure (p) and wall tension (t) in a hollow body defined by radii of curvature r_1 and r_2:

$$p = t\left(\frac{1}{r_1} + \frac{1}{r_2}\right)$$

which is simplified to $p = 2t/r$ and $p = t/r$ for a sphere and an infinitely long cylinder respectively. Therefore, the larger the enclosed cavity, the greater is the tension required in the wall to maintain the same pressure. Without making explicit use of this law, but essentially applying the same principle, Burch *et al.* (1952) derive that in the course of ventricular systole, although the pressure against which the heart contracts increases, the force required of the heart becomes less, because the smaller heart works at a better mechanical advantage. At the same time, of course, as the "leverage" increases, a greater amount of shortening of the musculature is required to displace the same volume. The systematic and accurate development of these matters seems urgently needed, and is likely to affect the appearance of problems such as Starling's law and efficiency (e.g. Burton, 1957, p. 808).

The recognition of another interesting feature is due to Woods (1892). He observed that there is a relation between wall thickness and curvature at each point in the ventricular part of the heart, which is in quantitative accord with Laplace's Law; the wall thickness in the left ventricle is about 6 or 7 times greater than in the right, where the pressures are correspondingly smaller. The same law also permits us to calculate the force which must be exerted by the ventricular wall to overcome the blood pressure. This, for the human heart, is found to be of the order of 5×10^5 dynes per cm.^{2} (Burton, 1957), which is 4 to 10 times lower than the values considered characteristic for warm-blooded striated muscles. Our actual measurements on the active tension in the cat's papillary and trabecular muscle give even lower values, about 2×10^5 dynes per cm.^{2} (compare Fig. 3). One may suspect the

possible existence of a regularity whereby smaller hearts have a weaker musculature, because of the smaller radii of curvature. Alternatively, of course, nature might have chosen to use thinner walls, and of this, examples might likewise be found. It might be observed here that even the skeletal muscles alluded to would not seem to have reached the maximally possible contractile force. There seems to be no good reason why the strength of muscle should not approach e.g. the tensile strength of tendon (apart from its "dilution" with metabolically active protoplasm), yet this is not the case except perhaps in unusual examples such as the muscles involved in the jumping of locusts.

That cardiac muscle has inherently a lower contractile strength may also be related to the finding that in extracted muscle preparations that contract upon the addition of ATP (Szent-Györgyi fibers), those derived from the myocardium (Taeschler and Bing, 1953; Benson *et al.*, 1958) display a considerably lower tension. Also, the frequently observed fact that the adenosinetriphosphatase activity of cardiac myosin is much lower than that of skeletal muscle myosin may or may not be related to this.

These few introductory remarks hardly touch the surface of cardiac physiology, but they must suffice here to indicate some of the numerous special problems that arise from the special physiological architecture of the heart, even when only its muscular elements are considered.

IV. INOTROPIC EFFECTS

One chapter of cardiac physiology, which will be considered in greater detail because of its close connection with the general physiology of muscles, is that of the various factors which control the strength of its contraction under experimental conditions, particularly in relation to the frequency of stimulation. This special class of inotropic effects has been studied for almost a century, and has recently been brought back into prominence by the work of Hajdu and Szent-Györgyi described below. The field was initiated by the classical studies of Bowditch (1871) on the staircase effect of the frog heart, whereas among subsequent investigations, an important study by Woodworth (1902) on the apex of the dog heart must be mentioned. The significance of the latter object is that it is demonstrably free of ganglion cells and, after considerable disagreement on this matter in earlier periods, it became the accepted opinion that the effects to be described are strictly a property of cardiac muscle and not of nerve elements. We

shall reconsider this crucial point later on. Phenomena of this nature are not unknown in skeletal muscle, but do seem to be less pronounced. A "staircase" ascent after a period of rest is frequently present, but the regular dependence of contractile strength upon frequency in a regular series of twitches is not observed in the muscles commonly studied. If this impression about such a difference were real, it might be related to the circumstance that many skeletal muscles do not move uninterruptedly in regular rhythms as the heart does, so that for the heart a change in the frequency of stimulation would correspond more uniquely to a definite physiological situation likely to cause an adaptation. However, such functional implications depend on the question to what extent these frequency-determined inotropic reactions occur *in vitro*; this is not clear. On the one hand, there is evidence that the staircase effect in the frog heart is suppressed by normal constituents of the blood (Hajdu and Szent-Györgyi, 1952a, b), so that it would play no role in the normal physiology of this animal. Yet, with mammalian preparations, the phenomena are regularly obtained in blood or serum. Direct observations on extra-systolic potentiation and other effects *in vivo* appear in the literature, but it is usually difficult to evaluate whether they are due to the mechanisms discussed herein.

The first manifestation, known as the staircase[1] effect, may be described in two ways. When a heart preparation, such as a frog ventricle or a dog ventricular tip, is kept quiescent after isolation, and stimulation is then begun, it is seen that contractions start weakly and increase stepwise to their final level. On the other hand, when a rhythmical stimulation is maintained at a regular rate, it is found that the strength of the contractions depends on the frequency of stimulation, in the sense that at a higher frequency the mechanical activity is stronger. Upon changing the frequency, the level of contractions corresponding to the new rhythm is reached after a few transitional beats. Thus, it is seen that the original staircase effect is merely a special case of a general phenomenon, the frequency-determined inotropy (Fig. 4). It is not superfluous to emphasize that these alterations in response in no way contradict the all-or-none law which was discovered by Bowditch in the course of the same work. This law was illustrated by Woodworth as follows: "A series of contractions was first produced by

[1] The German word for stair is Treppe. This term has gained acceptance in the English language literature, but, for proper use, it should be capitalized, and it should not be misspelled or mispronounced.

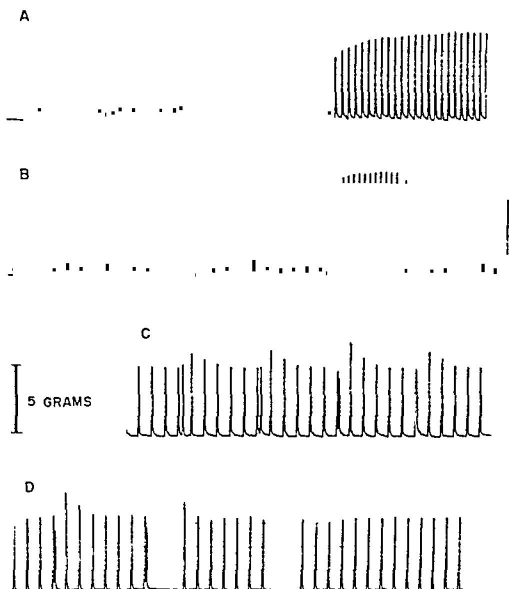


FIG. 4. Frequency determined inotropic effects of cardiac muscle. Original records obtained on a papillary muscle of the rabbit, about 3 mm.² cross section, at 27°C. in oxygenated Ringer solution. Calibration: 5 g. tension. Basic rate of stimulation: one per 5 sec.

A. The development of contractile strength (the Bowditch staircase) after a complete rest of 15 min. and of 4 min., respectively. Note that the first twitch has the character of a "rest contraction," more so after the shorter than after the longer rest.

B. Dependence of contractile strength upon frequency (the Woodworth staircase). Increase in frequency from the basic rate to 1 per 2 sec., and return to 1 per 5 sec.

C. Post-extrasystolic potentiation. When an extra systole is elicited 2, 1, and 0.7 sec. after a regular contraction, the potentiation of the following twitch increases. How- [illegible]

[illegible]

and recovery after the rest contraction is an expression of the Bowditch staircase.

minimal stimuli. When the height of contractions was well established, the intensity of the stimuli was suddenly greatly increased. After some time, the intensity was reduced to its former point. But no changes in the height of contraction were produced by these changes in the stimulus." The all-or-none principle only states that by an adequate stimulus the reacting tissue is either not at all, or else completely, activated to full response, and that graded stimuli do not cause graded responses. But the extent of the maximal response is determined by many additional parameters affecting the tissue's reaction. The staircase phenomenon thus illustrates that the response of the tissue depends among others on the repetitiveness of its activation; thus, the staircase phenomenon represents an "accumulative action of the contraction" (Dale, 1932). In other words, a contractile event, or the stimulus which elicits it, temporarily creates a condition in the muscle which predisposes it favorably for the next event of activity. That this condition may be based upon the presence of a substance was clearly formulated by Joffé (1931), who demonstrated that the inotropic changes in a donor heart could be humorally transmitted onto a second heart perfused in series; he held lipid substances responsible for this effect.[2]

Investigations on the staircase effect have continued through the literature (Kruta, 1937), and special mention must be made of recent investigations employing the papillary muscle of the cat's heart (Cattell and Gold, 1941, 1955). This material, because of its thinness and, therefore, its access to oxygen, has technical advantages over other mammalian heart preparations which would either require elaborate perfusion techniques, or might involve greater damage as a result of dissection. However, similar investigations by Whalen (1958a) on the papillary and trabecula carnea preparations also revealed a complication which may greatly affect our interpretations of many findings in this field. It was observed that a sudden increase in stimulus intensity is followed by pronounced inotropic changes. Dependent on the species from which the tissue was taken, these could consist of either a significant increase or a decrease in contractile strength, or a combination of both effects. We subsequently found (Brady *et al.*, 1959) that a train of stimuli immediately following a regular beat, to which the heart muscle shows no direct response due to its refractoriness, like-

[2] In view of our later remarks on epinephrine and norepinephrine, it must be stated that these catechol amines may accompany lipids during extraction procedures (cf. Von Euler, 1956).

wise produces strong potentiation. We believe that these effects are to be ascribed to the stimulation of nervous elements which produce epinephrine or norepinephrine (probably the latter), or acetylcholine, as the case may be. This conclusion is supported by the disappearance of this voltage-induced effect, after denervation of the heart (Whalen *et al.*, 1958) and after exhaustion of the norephinephrine supply subsequent to the application of reserpine (Whalen, 1958b).

These results by Whalen contradict the findings of Woodworth quoted above, that changing the intensity of stimulation caused no alterations in the strength of the response. The old literature contains many discussions about the possible participation of neurons in these responses. Woodworth's experiments, in conjunction with histological evidence, could be taken as an indication that, in his object, nerves were indeed absent, so that both the staircase effect and the potentiation phenomena to be discussed below can, in principle, be revealed by the myocardium itself. But his observations can equally well be interpreted by the assumption that in his case, the sensitivity of the nerve endings present was such that they were already maximally stimulated at the threshold of the myocardium, whereas they were not in Whalen's experiments, due to differences in the mode of stimulation. In either case, the observations on the high voltage effect and on the influence exerted by trains of stimuli do not affect the all-or-none doctrine. They merely are due to the neurogenic formation of substances changing the contractile strength of the muscle.

A second effect discovered by Woodworth, and studied recently by Wiggers (1925), Siebens *et al.* (1955), Cattell and Gold (1955), Garb and Penna (1955), Penna and Garb (1956), Rosin and Farah (1955), and by Hoffman *et al.* (1956), is that an extra systole interspaced within a regular sequence strengthens the following one or several beats (Fig. 4). This is the more pronounced, the closer the extra beat is spaced to its predecessor, although by this crowding, the extra contraction itself becomes smaller. Only when the extra systole becomes very small does the potentiation decrease again (Abbott *et al.*, 1959). This is taken as illustrating the promoting effect of the pause intervening between the extra beat and the first subsequent contraction. The positive inotropic influence caused by the extra systole, called the post-stimulation potentiation, can extend over several subsequent beats and can be preserved in the muscle during an interspaced period of rest, persisting for several minutes. Finally, a period of rest can, by itself, cause signifi-

cant augmentation of the following responses (rest potentiation). These various effects, which are not regularly accompanied by obvious changes in the action potential (although in our experience potentiation is sometimes correlated with a definite lengthening of the trans-membrane potential), can show themselves in several possible combinations. A phenomenon of clinical interest to which Garb and Penna have drawn attention is that of bigeminal rhythm, in which each second beat can be so weak as to be unable to open the aortic valve. Yet, such a beat need not be wasted, as it may condition the myocardium to strengthen its subsequent contraction; of course, the second beat is strengthened also because of the meanwhile increased diastolic distension. Similarly, strong beats in abnormal rhythms, e.g. the Wenckebach phenomenon, may be considered.

While thus far the observations have been consistent, it must be mentioned that changes can occur in the opposite direction, in the form of a negative Treppe, in which case also the other potentiation phenomena are reversed or absent (Whalen *et al.*, 1958).

The revival of interest in these inotropic phenomena is in no small measure due to the contributions by Hajdu and Szent-Györgyi (1952a, b), Hajdu (1953), and Szent-Györgyi (1953), who made the first attempt toward a penetrating understanding of its mechanism. These authors studied the frequency dependence of the strength of contraction of the frog ventricle under a variety of conditions, and found this dependence to be abolished by serum and by several steroids such as deoxycorticosterone and progesterone in high concentrations. Aldosterone has not yet been studied in this connection, although its relation to ion retention problems would make it of great interest in view of the interpretation offered below. Recently, Hajdu *et al.* (1957) have identified as β-palmityl lysolecithin a substance with similar action isolated from acetone extracts of mammalian tissues (notably present in heart, liver, plasma, and especially in adrenal medulla; absent in adrenal cortex, erythrocytes, and muscle). Similarly, digitalis glycosides depressed the staircase effect, and the concept was developed that these drugs might act by restoring those properties of the heart normally maintained by physiological substances. The applicability of this concept to mammalian heart muscle remains to be studied since, as pointed out, its inotropic reactions persist in the presence of blood.

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occurrence and role of nerve elements within the myocardium, which have already been alluded to. In a significant study, Middleton *et al.* (1956) propose that several responses of the heart depend on the presence of nerve. It is true that in many areas of the heart ganglion cells are supposed to be absent, which was also Middleton's experience with regard to the papillary muscles employed in their work. Yet, recent studies (Meyling, 1953) with special staining techniques have revealed the occurrence of nerve cells peripheral to autonomic postganglionic neurons in many areas of the body. At any rate, the work by Middleton *et al.* showed that, while in a nontreated papillary muscle preparation acetylcholine had variable inhibiting or enhancing effects, the same substance invariably strengthened contraction after atropinization. Nicotine suppressed this potentiating effect, after exerting a transient activation by itself. These and other observations seemed to leave only the interpretation that acetylcholine exerts two separate inotropic actions: a muscarinic depressant effect acting directly upon the muscle, and a nicotinic stimulating effect which operates indirectly by the release of sympathin from nerve elements.

In view of these facts, we have examined whether the role of the potentiating substance could not be ascribed to norepinephrine. In one regard, a clear answer was obtained (Whalen, 1958a): after cardiac denervation and similarly after application of reserpine, the high-voltage induced potentiation disappears completely. This, then, is clearly explained by stimulation of sympathetic fibers releasing, presumably, norepinephrine. Our results with trains of impulses are likwise ascribed to the stimulation of nerve. With regard to the other effects, however, the results have not been unequivocal, and seem to depend greatly on the time interval after denervation. While further work is required, for the moment there is no support for the assumption that norepinephrine is solely responsible for the majority of the effects, and we take the view that it is not.

The described facts have exposed the inotropy of cardiac muscle as a complex phenomenon which seems explainable, on the one part, by the balance between inflow and outflow of cations, while on the other hand, indications press for the postulation of a potentiating substance. Upon further investigation, these views may not be irreconcilable. We should furthermore consider that the potentiating substance does not have to be of the nature of a hormone or neurohumoral agent, but may be a critical metabolic intermediate, which in a regular state of rhythm-

the resting heart accumulates potassium, but that some is lost in each contraction (see Section III), so that the intracellular potassium level depends on the respective magnitudes of loss and reabsorption in a quasi-stationary state. *In each contraction, about 127 μeq. of potassium* permeate outward, which can be estimated to be about 20 μμM per cm.[2] cell surface, ten times higher than in nerve. It may be asked whether this reflects merely a difference between nerve and muscle cell membranes, or whether the potassium release is correlated with the activation phenomena of the contractile matter, in addition to its role in membrane depolarization. Hajdu (1953) and Szent-Györgyi (1953) have advanced the theory that this potassium release is the direct cause of the staircase phenomena, acting by the following mechanism. The high potassium concentration associated with the resting state is unfavorable to the contractility of the actomyosin system, while its reduction by about 3%, as was demonstrated to occur at the optimal rate of stimulation, gives rise to a favorable composition of the intracellular medium. This proposal is in full accord with the extremely sharp dependence of the contractility of actomyosin threads upon the potassium concentration.

In studies along similar lines, Moulin and Wilbrandt (1954) and Niedergerke (1955) have called attention to the distribution of calcium instead of potassium. These explanations are not unrelated, in view of the manifold interactions between potassium and calcium effects. In the investigation by Rosin and Farah (1955), however, it was pointed out that the potassium-release theory cannot account for the maintenance of the contraction potentiation over a rest period, nor for the rest potentiation itself, and that the assumption of a potentiating substance is required to explain the entire group of phenomena with one single formulation. Hajdu (1956) has defended his position by stating that his theory was directed at an explanation of the staircase effect only, and should not be discarded because it fails to explain cases for which it was not intended. This distinction would be satisfactory unless one holds that all the mentioned effects are so closely related as to require one single explanation. We feel that the mere assumption of a potentiating substance does not yet provide such a unitary explanation, but merely shifts the difficulty because it now becomes necessary to explain why it is enriched in such a variety of conditions.

The final judgment on the mechanism of the frequency-induced inotropic effects will also greatly depend on the decisions regarding the

should not concern us greatly in this connection, because reversibility in heat engines is an abstraction which in practice one may try to approach as well as possible but will never reach. The value of the concept does not suffer thereby.

While in the case of a heat engine, the efficiency has a direct theoretical basis, we are in a more difficult position when we consider a case like muscle, where a number of chemical reactions occur, one of which presumably engages in some form of mechanochemical linkage with the working substance. Here, the factor Σ (ΔH) contains the chemical contributions of many reactions which are not directly concerned with the contraction process. Some of these, e.g., might be heats of neutralization. In a heat engine, this would not matter, since any form of heat conferred upon the working substance would do; but in a chemodynamic engine, only the energy of the directly coupled reaction is relevant, and any extraneous reactions would merely exaggerate or reduce the energy seemingly available to the engine, and thus lower or raise the apparent efficiency.[3] These limitations must be kept in mind when efficiency figures obtained from physiological experimentation are to be evaluated.

It should not be concealed that there is serious question as to the fundamental validity of the use of "efficiency" as defined in the present context. As applied to heat engines, the concept has a fundamental meaning, since it is immediately derived from the transfers of heat at two temperatures in the engine cycle. But in a chemodynamic engine, there are no such heat transfers, and hence the concept is deprived of its real basis. Alternatively, one could consider a "free energy efficiency," $\varphi = W/\Sigma(\Delta F)$, which would be a measure of the perfection with which the available free energy is utilized. If the fundamental chemical event were known and isolated, its efficiency $\varphi = W/\Delta F$ would approach unity, but for avoidable or unavoidable dissipative losses. The difficulty is that, in general, the free energy values are less accessible to us than the enthalpy values. Fortunately, when considering the total respiratory metabolism of an organ, the difference between ε and φ is not appreciable, because for reactions such as respiration with large energy effects, the values for ΔF and ΔH are always closely similar. Hence, statements about the efficiency of muscle or (see below) heart are

[3] It is not our intention to introduce "apparent efficiency" as an official term. Yet, it may be of use in this chapter to indicate the efficiency value obtained by global application of the formula $\varepsilon = W/\Sigma(\Delta H)$.

ic activity may be in somewhat short supply, and would accumulate to an optimal concentration during a brief rest. As an example, we may refer to the work of Ellis (1955), on the correlation between hexose monophosphate level and contractile strength.

The final identification of the substances regulating contractile strength must strongly influence our judgment as to whether the staircase effect and related phenomena do occur *in vivo*, physiologically or pathologically, apart from those uncertainties already alluded to that may be ascribed to differences between frog and mammalian heart. The main point is, that if under experimental conditions in mammalian heart preparations, the effects are caused by the stimulation or overstimulation of nerve elements, they may not at all occur *in vivo* in the autonomously beating heart in response to its change in frequency (and so, among others, play no role in the changes occurring after an extra systole, or in the bigeminal or Wenckebach rhythms). This would not diminish the significance of studies in this field, but shift their importance to a different aspect: the inotropic effects of norepinephrine, epinephrine, and acetylcholine, released by the cardiac nerves or in response to stress situations. If, as we believe on the other hand, the phenomena reflect inherent mechanisms of the myocardial fibers themselves, the frequency-determined inotropy would have direct physiological significance as such.

V. THE EFFICIENCY OF MYOCARDIAL ACTIVITY

When relating the amount of work performed by the mechanical activity of a muscular organ to its total energy liberation, the term "efficiency" is used in its practical or engineering sense: $\varepsilon = W/\Sigma(\Delta H)$, or, the efficiency equals the work divided by the sums of the heat effects of all the associated chemical reactions. The thermodynamic justification of its application to muscle contraction is less obvious than it seems, for several reasons. We shall only present the following considerations. In one case, that of a heat engine operating e.g. in the Carnot cycle, the meaning of the efficiency or conversion factor is theoretically defined by the equation $\varepsilon = (T_1 - T_2)/T_1$, where T_1 and T_2 are the temperatures of the warmer and the colder reservoirs, respectively. The validity of this formula rests on two conditions: that the arrangement is that of a heat engine, and that it works perfectly reversibly. None of these conditions may be assumed to be fulfilled in contractility. That the muscle engine, in order to reach finite speeds, may not work reversibly

and it is doubtful whether as clear a distinction will be found here between initial and recovery heat. However, the essential feature of the recovery heat is not that it is delayed, but that it represents a sequence of biochemical reactions not directly connected with contraction, namely oxidative phosphorylation and a reversal of whatever acid-base shift may have occurred in the more primary chemical reactions. In this sense, we can undoubtedly speak of "initial reactions" and "initial heat" for the heart even if they are not resolved in time, and so the intrinsic efficiency may, again, be 40% or better.

While this has not yet been established for the heart, skeletal muscle has the feature that, while a certain heat of activation (maintenance) and of internal shortening appear in any case, any work done is performed not at the expense of these heats, but at the expense of energy mobilized in addition (the "Fenn effect"; Fenn, 1923; Hill, 1938). Conversely, work done upon a muscle during its activity does not appear as heat, but is stored in some other form (Abbott and Aubert, 1951). It might be argued that activation and shortening, on the one hand, and work on the other, are independent processes, and that fundamentally work is performed with perfect efficiency. However, our knowledge of the coupling mechanisms is insufficient to allow judgement as to whether such a viewpoint is fruitful or correct.

The maximal efficiency of the heart is reached only under certain optimal conditions of work and load, and a great deal of effort has been spent on investigating the connection between work and oxygen consumption. One of the first important generalizations was that of Visscher and Starling (1927) to the effect that the oxygen consumption is directly determined by the diastolic fiber length so that, since also the work per ejection is determined by that length, the efficiency relations would follow from these two parameters. However, further developments in this field have been far from clarifying, and reference must be made to a recent symposium (Katz, 1955; Sarnoff, 1955; Rushmer, 1955a, b) for the latest discussions.

A reduction in efficiency is accepted to be the underlying mechanism in cardiac failure. This has recently been demonstrated in a most convincing manner, both for the hypodynamic state of an isolated perfused heart (Lorber, 1953), and for the failing heart in human patients (Bing *et al.*, 1949; Blain *et al.*, 1956). In both cases, the performance decreases without change in oxygen consumption. The two demonstrations differ, it is true, in that the human heart *in vivo* maintained its workload at a

fairly exact, regardless of the fundamental problem of a valid definition.

The apparent mechanical efficiency of skeletal muscle was thoroughly studied among others in the classic paper by Hill (1939) on the frog sartorius. The experimental approach was to measure the work performed under the best possible conditions and, simultaneously, to measure the heat Q produced. The efficiency would then be $\varepsilon = W/(W + Q)$. This efficiency was found to be highest at a certain speed of shortening—a relation to be discussed below—and was then found to be slightly more than 40%.

In order to evaluate the meaning of this result, it must be kept in mind that in this experimental approach, Q measures only the initial heat of the contraction. Since the recovery heat is about equal to the initial heat, it follows that $\Sigma(\Delta H)$ for a complete activity cycle would be about twice as large, and the apparent efficiency about 20%. These figures illustrate the relative meaning of the concept in this case. From the point of view of the total energy balance of the organism, 20% is the significant number because it indicates the metabolic "cost" at which work can be obtained. Yet, with regard to the intricate mechanism, it is less applicable and suggests too low a figure for the effectiveness. Clearly, the restitution reactions, which occur afterwards, are not directly involved in the contraction process, and so the 40% figure is more meaningful. Even this is not absolute, because, in the transient events during contraction, heats of neutralization might appear which are not relevant to the issue. Only one negative conclusion arises: that muscle cannot be a heat engine, because in that case it could reach such a high efficiency only with the help of very high and entirely impossible temperatures.

The efficiency of cardiac muscle has not been studied in the same fundamental manner. Yet, by estimating cardiac work together with oxygen consumption, and evaluating the latter in terms of heat production with the formulas in use for the study of energy metabolism, reasonably good estimates have been obtained (Evans and Matsuoka, 1914). Recently, such studies were performed by Bing *et al.* (1949) in the intact human body with catheterization technique. In close agreement with the studies on skeletal muscle, the efficiency amounts to 20–25%, but is markedly lower in congestive heart failure, which is considered to be a disease of the energy utilizing mechanisms. Direct myothermic studies on cardiac muscle preparations have not yet been possible, for technical reasons (see Fischer, 1927 for an early effort),

and it is doubtful whether as clear a distinction will be found here between initial and recovery heat. However, the essential feature of the recovery heat is not that it is delayed, but that it represents a sequence of biochemical reactions not directly connected with contraction, namely oxidative phosphorylation and a reversal of whatever acid-base shift may have occurred in the more primary chemical reactions. In this sense, we can undoubtedly speak of "initial reactions" and "initial heat" for the heart even if they are not resolved in time, and so the intrinsic efficiency may, again, be 40% or better.

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greater diastolic filling whereas the isolated heart did not; whether this is a significant distinction or not is a matter of dispute among the authors (Lorber, 1953; Blain *et al.*, 1956; Bing, 1955a, b). During the last years, the concept has arisen that cardiac failure is a "disease of the energy-transfer mechanism" (Olson and Schwartz, 1951; Bing 1955a, b; Wollenberger, 1949). This view is based on the above-mentioned reduction in efficiency combined with the observation that in a failing heart the content of high-energy phosphate compounds are not diminished (Wollenberger, 1949). It must be mentioned, however, that one exception has been reported (Greiner, 1952), in which the ATP content of papillary muscles in the hypodynamic state was decreased, and was restored to normal by ouabain.

Besides this pathological variation in efficiency, it is also held that physiological "energotropic" effects exist, in that acetylcholine improves, and epinephrine lowers the efficiency (Gollwitzer-Meier, 1938). Such effects are certainly not impossible; there is no reason why the efficiency under otherwise comparable conditions should not depend on the various biochemical and mechanochemical coupling phenomena that constitute the contraction cycle, and why these phenomena should not be influenced by regulating substances, like e.g. thyroxin is believed to cause uncoupling of phosphorylation from respiration. For example, it is known that sympathomimetic substances affect phosphorylase (Sutherland, 1956), in a way which can lead to an accumulation of hexose phosphate (cf. Cori and Cori, 1936), and these in turn have been correlated with changes in contractile strength (Ellis, 1955). Also, it has been found that epinephrine greatly increases the respiration of resting as well as active cardiac muscle (Whalen, 1957). Several authors have discussed these energotropic effects (Eckstein *et al.*, 1950; Gauer and Kramer, 1939; Gremels, 1936; Gremels and Zinnitz, 1937–1938; Raab, 1956).

An attempt has been made to show that the energotropic effects of acetylcholine and epinephrine are not independent phenomena, but that they can be derived from the inotropic activities of these substances (Schumann, 1950, based upon measurements by Reichel, 1939a, b). It is proposed, e.g., that when a contraction is potentiated by epinephrine, more power is generated than is utilized, hence the efficiency is lowered. This explanation is not, as has been stated (Raab, 1956), invalidated by the observation that after epinephrine the coronary venous blood becomes warmer, because it is clear that any decrease in

efficiency, regardless of its mechanism, must produce more waste heat. On the other hand, we have not become convinced that the explanation, in the form in which it is given, is based upon sound cardiodynamic considerations, although elements of it may be retained when more is known of the relations between dynamics and efficiency along the lines indicated below.

A very fundamental question regarding the efficiency of the heart can be raised, starting from the consideration that skeletal muscle has an optimal efficiency at a certain velocity of contraction. We shall explain the connection between efficiency and velocity by means of schematic diagrams. Figure 5a schematically shows a load-efficiency

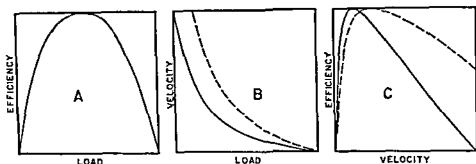


FIG. 5. Diagrams illustrating the derivation of the velocity-efficiency relation from the more familiar load-efficiency and load-velocity relations. The diagrams are idealized, and are based upon the parabolic load-efficiency curve obtained with tetanized frog sartorius muscle. A: dependence of efficiency upon load; the efficiency is zero when no load is lifted or when the load is too heavy to be lifted, and reaches its optimum at a medium load. B: the load-velocity (or force-velocity) curve and (broken line), the same curve for a muscle assumed to have the same maximal strength (P_o) but twice the intrinsic velocity. C: the velocity-efficiency relation obtained from A and B coordinate transformation. It is seen that the faster muscle reaches optimal efficiency at a higher velocity of shortening.

The transition of a slower into a faster muscle occurs during the frequency-determined inotropic strengthening of heart muscle. It is uncertain whether in this case P_o, which has not yet been determined, remains unchanged, but similar relations occur when both load and velocity increase. However, mere increase in P_o without changing the intrinsic velocity does not shift the velocity of optimal efficiency.

diagram. Such a diagram would ideally be based upon measuring (Hill, 1939) the work performed as well as the total or initial energy mobilized (total or initial heat plus work), but the general course of the curve can be understood by reference to the fact that no work is done with either zero or maximal load, and that the shape of the bell-shaped curve is furthermore influenced by the shortening heat and the length-tension diagram. Figure 5b shows a load-velocity curve. It is clear

that by a transformation of coordinates one can combine these diagrams into Fig. 5c, showing the dependence of efficiency upon velocity of shortening, and revealing the sharp optimum in this relation. It appears to be accepted that for skeletal muscles at a given temperature this optimal velocity is constant.

Would such a constancy be compatible with the function of the heart, which may have drastic changes in output required from it, and which will meet these requirements in part with a higher frequency and a greater velocity of shortening? It is obvious from Fig. 5c that e.g. a threefold change in velocity on the same curve might bring a muscle from an optimal to a very low efficiency. It has been discovered, however, that one aspect of the staircase effect consists of a profound change in the load-velocity relation, such that at a higher frequency of stimulation the muscle becomes not only stronger but also faster (Abbott and Mommaerts, 1958). This would mean that in the velocity-efficiency curve (Fig. 5c), the optimum would shift to higher velocities, and hence that the heart adapts itself to the new situation by having its optimal efficiency at greater contraction speed. The possibility of this adaptation, which may be of fundamental physiological importance, was anticipated by *A. V. Hill (1957): "... the possibility remains that* cardiac muscle may be able to change its intrinsic speed according to the conditions: the question suggested is whether the heart, unlike the skeletal muscle, becomes intrinsically quicker when the frequency of its beat rises." It is seen that this prediction seems to have been confirmed. Actually, the changed time relations during the staircase effect were observed long ago by Hofmann (1901) but the significance of this phenomenon, observed again by Niedergerke (1956) and by Abbott and Mommaerts (1958), was not appreciated until Hill raised the question. A direct experimental study of the efficiency of the papillary muscle, analogous to that on the frog sartorius, would be of great importance, but has not yet been carried out because of the difficulties of myothermic measurements on myocardial preparations.

Space limitations forbid a discussion of cardiac metabolism, but reference may be made to a recent chapter devoted to that problem (Mommaerts, 1958).

VI. SOME FUNDAMENTAL ASPECTS OF CARDIAC CONTRACTILITY

In every contractile tissue, activity is caused by an event of stimulation. Between this and the observable mechanical processes, there

must be a considerable chain of events, which are entirely unknown, but which are just beginning to be subjected to investigations. Most of these attempts were undertaken with skeletal muscle, and will be referred to elsewhere in this book. However, the results of the current work by the authors (Abbott and Mommaerts, 1958) suffice to permit a brief discussion of the influence of these developments upon cardiac physiology.

It is likely that the link between stimulation and contraction is of a multiple nature, and it is possible that several of the connecting steps are independently linked with metabolic reactions. This is suggested e.g. by the facts that processes resembling activity metabolism can be elicited without activity (Hill and Howarth, 1957), and that the shortening can be abolished without eliminating the activation heat (Hill, 1958). So far, however, the available knowledge regarding precontractile events is mainly of a mechanical nature, and applies especially to the change immediately preceding contraction or isometric tension development, which is called the active state. We shall define this as a condition in which the contractile sections of the myofibrils are able to exert tension to the full degree possible under the given internal and external conditions, and to undergo shortening at a velocity characteristic for the given muscle under those same conditions. It is found that in skeletal muscle, this active state is established very soon after stimulation, persists for a definite time at a constant plateau, and then declines (Hill, 1953). In tetanus, it is maintained constantly at that same plateau, as long as the stimulation is applied.

The implications of this definition are best illustrated by discussing the genesis of an isometric twitch (Fig. 6). While the active state, as said, is established rapidly, externally observable tension develops only with a marked delay. This is so for two related reasons, both due to the fact that the potentially existing tension must first transmit itself through the series elastic component to allow its macroscopic manifestation. Initially, as revealed by the slope of the onset of the tension curve (phase 1), this is limited by the maximal speed of shortening of the contractile component, which effects the stretching of the series elasticity needed for this manifestation of tension. However, as this proceeds, the opposing force exerted by the elastic component increases, and correspondingly, because of the force-velocity relation, further shortening of the contractile component and hence further development of tension decrease in rate (phase 2). Finally, a point is reached

at which the active state declines while the external tension is still waxing; when these two tensions are equal (phase 3), there is no more shortening of the contractile component, and the isometric tension curve has reached its peak.

It may be instructive to compare this relation (the mechanisms of which are unknown) with chemiluminescence or with phosphorescence. Here, a molecule A is brought into an excited state A*, either by the energy effect of the associated chemical reaction, or by the

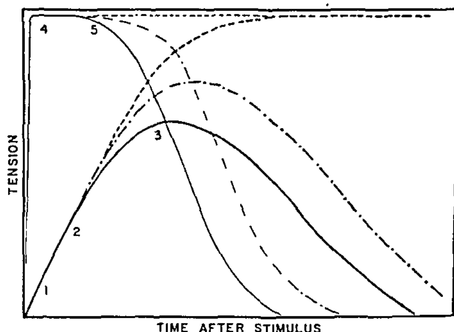
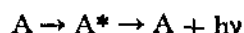


FIG. 6. Relation between active state (light curves) and contraction (heavy curves). Explanation of points 1, 2, and 3 on the main (solid) curves in the text. The active state reaches its full amplitude almost immediately (at 4) and is maintained at its full activity until the beginning of its decline (at 5). The dash-dot curves refer to a case where the active state is lengthened and, as a result, the twitch becomes stronger and longer. The broken curves illustrate that in a tetanus the active state is maintained long enough to permit its full external manifestation as measurable tension.

It will be seen that, alternatively, a twitch could also be strengthened by increasing the intensity of the active state with a proportional increase of the initial velocity of tension development, or by an increased velocity by itself.

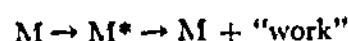
previous absorption of light. From the excited condition, the molecule then falls back to the ground state, under the emission of light:



$$A \rightarrow A^* \rightarrow A + h\nu$$

Similarly, it might be proposed that the responsible parts of the muscle structure M are brought into the active state M* by a chemical re-

action, and return to the ground state M under the "emission" of mechanical energy (Mommaerts, 1951):



$$M \rightarrow M^* \rightarrow M + \text{"work"}$$

As in chemiluminescence and phosphorescence, the reaction $M^* \rightarrow M$ has a characteristic time course, which may be considered responsible for the limitation on the possible velocity of shortening. This formal analogy should not be taken too seriously at the moment, for lack of sufficient information. One special feature, without direct analogy with phosphorescence, should be indicated. The condition M^* has already an altered mechanical property, namely that of instantly exerting tension.

No universal method for directly measuring the full course of the active state has yet been developed, but three major approaches exist whereby several features may be characterized. The first of these is that the tetanic tension itself directly measures the intensity of the active state. This method is not applicable to cardiac muscle. More precisely: the myocardium can be tetanized only under very special conditions (Whitehorn, 1954), so that the intensity of the active state can be so measured only under these conditions, and not in the more important normal cases. Secondly, the plateau can be measured, and the early onset demonstrated, by applying a rapid stretch soon after the stimulus (Hill, 1950). This extends the series elasticity by the required amount and so permits the full manifestation of the active state unlimited by the velocity of shortening. This has been applied by us to myocardial preparations (Abbott and Mommaerts, 1958) but the results were completely unexpected and have not yet been interpreted. The third method, due to Ritchie (1954), is designed to measure the decline of the active state. It consists of quick releases applied at various intervals after the stimulus, sufficient to permit a shortening of the series elasticity, so that the twitch must start anew. A series of twitchlike tension curves is so obtained (Fig. 7), the peaks of which lie on the declining active state curve. This method has been used successfully by us on cardiac preparations (Abbott and Mommaerts, 1958), and several applications will be given below. It was found that the decline curve is steep (Figs. 7 and 9); this, incidentally provides a sharp explanation as to why the heart cannot be tetanized; normally the active state is over while the refractory period still persists.

From these explanations, it may be derived that variations in the

intensity of a twitch can be due to several causes. It will be obvious from Fig. 6 that a prolongation of the active state, by allowing more time, will intensify the twitch. This is the explanation of Hill and Macpherson (1954) for the intensification of a muscle twitch by nitrate or iodide Ringer. The tendency in muscle research currently is to consider this as the major mechanism. The second major possibility is a variation of the intensity of the active state, which seems to be the mechanism involved in the fatigue of skeletal muscle (unpublished). Finally, as is

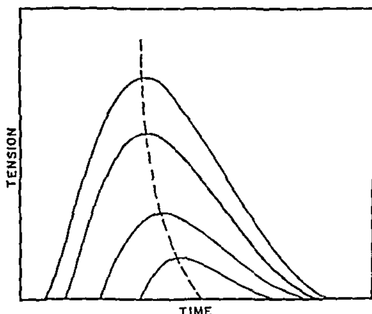


FIG. 7. Tension redevelopment curves of heart muscle obtained with the Ritchie method (see text). Solid lines. tracings of oscilloscope records made on a cat papillary muscle preparation; total duration of sweep, 1 sec. Broken line: resulting curve indicating the decay of the active state. Compare Fig. 9.

again obvious from Fig. 6, an increased velocity of shortening would result in a stronger twitch even when both the intensity and the active state remain constant. *Each of these variables; intensity and duration* of the active state and velocity of shortening, seems to be determined by the events interpolated between the stimulus and the final mechanical activity.

Our investigations on cardiac muscle have been directed towards an analysis of the inotropic effects described in the preceding section. Briefly, the following results emerge.

When, in the course of an experiment on the staircase effect, the contractile strength increases at a higher frequency of stimulation, two

different changes are obvious (Fig. 8). One is that the isometric tension increases; the other that the velocity of shortening increases, while at the same time the duration of the twitch becomes less. The active state is not prolonged but is, on the contrary, markedly shortened (Fig. 9). Certainly, then, potentiation of the contraction is not due to an extension of the active period. The two other mechanisms show interrelations which have not been completely elucidated. Studies on the force-velocity relation show, indeed, a marked increase in the velocity

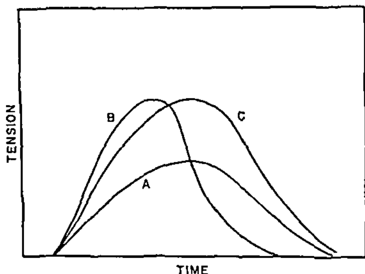


FIG. 8. Schematized, but authentic, twitch curves of a papillary or trabecular muscle preparation, A at low, B at higher frequency of stimulation. Total duration of sweep, 1 sec. The frequency-determined potentiation makes the twitch stronger, faster, and shorter. Curve C would indicate either a twitch potentiated by a preceding extra systole, or one obtained in the transition from low to high frequency when the higher level of contractility is reached but before the velocity changes are complete.

of shortening, so that this mechanism must be, at least, contributory. Whether the intensity of the active state changes has not yet been decided.

Niedergerke (1956), who already considered the concept of the active state into cardiac physiology, although without direct experimental observations, reports experiments on the negative staircase in the frog heart in the presence of elevated amounts of calcium. His results indicate that in that particular case, the strength of the contraction may be determined by variations in the duration of the active state. However, the more physiological variations operate through the mechanisms indi-

cated above. We believe it probable that in heart failure the amplitude of the active state is decreased, or the intrinsic velocity of shortening lowered, such that a hypodynamic state results.

The approaches indicated in this section are still very new, as far as cardiac physiology is concerned, but it is likely that they will have a considerable impact upon the development of this field.

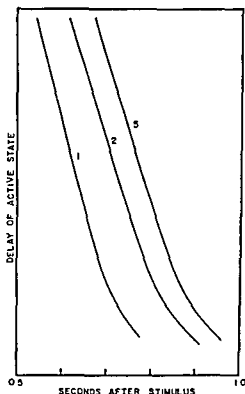


FIG. 9. Decay curves of the active state (compare Fig. 7) determined on a cat papillary muscle at 22°C. Intervals between stimuli 1, 2, and 5 sec., as indicated near the individual curves.

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